REMARKS

I. Status of the claims and Support for Amendment

Claims 53 and 54 are cancelled by the present amendment.

Claims 28–30 and 48–52 are amended and new claims 55–62 are added.

Claims 28–30, 48–52, and 55–62 are currently pending.

Support for the amendment of the claims is found in claims 34–39 as originally filed.

II. Rejection under 35 U.S.C. § 103

Claims 29, 30, and 48-54 (and presumably 28) are rejected as allegedly being unpatentable over the reference edited, not authored, by Dieter Adam (authored by Burzynski, S.R. *et al.* "Phase II Clinical Trials of Antineoplaston A10 and AS2-1 Infusions in Astrocytoma", in *RECENT ADVANCES IN CHEMOTHERAPY*, Eds. Dieter Adam *et al* (1991), referred to hereinafter as Burzynski *et al.*) for the reasons cited in the Office communication dated December 17, 2003. Specifically it is alleged that

> no unobiousness is seen in combining the compound of formula III and the compound of Formula IV together, since each is taught to be useful to treat cancer. Furthermore, once the usefulness of a composition is taught, it is within the skill of the artisan to determine the optimum dosages, rates of infusion and combined concentrations of compounds.

Applicant respectfully traverses.

As currently amended independent claims (claims 28 and 55) are drawn to methods requiring treatment of a patient with two independent compositions, designated a first pharmaceutical composition and a second pharmaceutical composition. In each claim the second pharmaceutical composition comprises a compound of formula I and a compound of formula III present in a 4:1 ratio, by weight. Furthermore, the compounds are present at a concentration of from about 200

mg/mL to about 350 mg/mL. Additionally, the claims require the second pharmaceutical composition to be delivered at a rate of from about 100 mL/hr to about 400 mL/hr.

There is nothing taught or suggested by the Burzynski *et al.* reference that would motivate one of ordinary skill in the art to provide the claimed method. In contrast, as was established in the parent of the current application (now issued as U.S. Pat. No. 6,258,849), the use of a composition of comprising a compound of formula I and a compound of formula III present at the claimed concentrations and ratios has surprisingly unexpected benefits. As evidence of this finding and for the Examiner's convenience, Applicant has attached copies of two 37 C.F.R. § 1. 132 Declarations prepared by Dr. Stanislaw R. Burzynski, filed during the prosecution of U.S. Pat. No. 6,258,849 (the §132 Declarations are respectively dated August 25, 2000 and January 18, 2001).

The August 25, 2000 §132 Declaration demonstrates that cancer patients experienced surprisingly high positive response rates to treatments using "concentrated" A10[1], especially in combination with AS2-1[2]. These response rates were not seen using a "diluted" A10 treatment (see paragraphs 16–20).

Paragraphs 6–12 of the Supplemental 37 C.F.R. § 1. 132 Declaration, executed January 18, 2001, further elucidate the surprising benefits experienced by patients treated with concentrated A10, but not those treated with dilute A10. These benefits include decreased fluid retention, enhanced elimination of waste chemicals, and better tumor penetration by the drug (*i.e.* A10).

Moreover, as pointed out in paragraph 5 of the January 18, 2001 Supplemental Declaration, it would not be obvious to one of ordinary skill to use higher concentrations of a chemotherapeutic drug because:

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[1] A10 is a composition comprising a mixture of phenylacetylglutamine (a Formula I compound) and phenylacetylisoglutamine (a Formula III compound) present in a 4:1 ratio, by mass.
[2] AS2-1 is a composition comprising a mixture of phenylacetate (a Formula IV compound) and phenylacetylglutamine (a Formula I compound) in a 4:1 ratio, by mass.

typically in the treatment of cancer, and other neoplastic disease, chemotherapeutic drugs are delivered in dilute solutions in order to minimize their negative effects to the patient. Contrary to conventional practice, which mandates using low concentrations of chemotherapeutic drugs, I have demonstrated the advantages of using concentrated A10 to treat neoplastic disease. It is my belief that applying such high concentrations of drug, as described and claimed in the instant application, is a completely new and innovative concept.

Additionally, work by Applicant has shown that there is improved efficacy for the patient when A10 is administered in conjunction with AS2-1. (See page 8 of the May 2004 report entitled by Waldbillig *et al.* entitled "*Uptake and intracellular binding of antineoplastic agents phenylacetic acid (PN) and phenylacetylglutamine (PG): Effects on epigenetic mechanisms of gene regulation and gene expression*", which was submitted to the FDA on May 26, 2004 (a copy of which is enclosed as part of the Information Disclosure Statement). Also enclosed as part of the IDS are an abstract of data and support material for a presentation to be made this month, September 9–11, 2004, at the "*World and Ehrlich Conference on Dosing of Antiinfectives*" in Nurenberg, Germany. The data to be presented in Nurenberg provide further support for the assertion that the currently claimed invention provides novel and unexpected results.

In view of the foregoing facts, it is Applicant's belief that there is nothing in the Burzynski *et al.* reference that would motivate one of ordinary skill in the art to make and/or use the currently claimed invention. Accordingly, Applicant believes that the rejection of the instantly pending claims, as being unpatentable over the Burzynski *et al.* reference, has been overcome and may now properly be withdrawn.

III. Conclusions

In view of the foregoing Amendments and Remarks, Applicant believes that objections to and rejections of the instant application have been addressed and overcome. Accordingly, Applicant respectfully requests favorable reconsideration of the case and issuance of a Notice of Allowance therefor.

In an effort to facilitate progression to grant, the Examiner is invited to contact the undersigned attorney at (713) 787-1589 with any questions, comments, or suggestions relating to the referenced patent application.

Respectfully submitted,

Matthew L. Madsen
Reg. No. 45,594
Attorney for Applicant

HOWREY SIMON ARNOLD & WHITE, LLP
2941 Fairview Park Drive, Box 7, Falls
Church, VA 22042
(713) 787-1400

Date: Sept. 8, 2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Stanislaw R. Burzynski

Serial No.: 09/121,567

Filed: July 23, 1998

For: TREATMENT REGIMEN FOR ADMINISTRATION OF PHENYLACETYLGLUTAMINE, PHENYLACETYLISOGLUTAMINE, AND/OR PHENYLACETATE

Group Art Unit: 1614

Examiner: J. Goldberg

Atty. Dkt. No.: BURG047/KAM
(10379.0047.NPUS00)

COPY

DECLARATION OF STANISLAW R. BURZYNSKI, M.D., PH.D.
UNDER 37 C.F.R. § 1.132

I, Stanislaw R. Burzynski, do hereby declare that:

1. I am a citizen of the United States and that my current residential address is 20 West Rivercrest, Houston, Texas, 77042.

2. I received a Medical Doctorate from the Medical Academy in Lublin, Poland, in 1967 and a Doctorate of Philosophy in the field of biochemistry from the Medical Academy of Lublin in 1968. I did my medical internship in internal medicine, surgery, pediatrics, obstetrics, and gynecology from 1969-1970 in pertinent departments at the Lublin Medical Academy. I did my medical residency in the Department of Internal Medicine of the Medical Academy in Lublin from 1969-1970. From 1970-1977 I was a researcher and Assistant Professor at Baylor College of Medicine in Houston, Texas. I am currently the Director and Chairman of the Board of the Burzynski Research Institute

which I have operated and directed since I founded it in 1977. I am the owner of the Burzynski Clinic which was founded in 1979.

3. I am the author or co-author of more than 170 scientific publications. I am a member of The American Association for Cancer Research, The American Medical Association, The American Chemical Society, The Harris County Medical Society, The International Union of Pure and Applied Chemistry, The New York Academy of Sciences, The Society for Neuroscience, The Southern Medical Association, The Texas Medical Association, The Society of Sigma Xi, The American Academy of Medical Ethics, and The World Medical Association.

4. I am completely familiar with the subject matter and disclosure of United States Patent Application Number 09/121,567 (hereafter called "the '567 application"), of which I believe that I am the first and sole inventor. I am generally familiar with the procedures used at the Patent and Trademark Office to consider and distinguish prior art references from pending patent application's claims. I am currently assisting with the prosecution of the '567 application.

5. As one of the co-authors, I am completely familiar with the reference entitled "Preclinical studies on antineoplaston A10 injections" by A.Q. Ashraf, M.C. Liau, M.O. Mohabbat, and S.R. Burzynski, *Drugs Exptl. Clin. Res. Suppl. 1,* 1986, 12:37-45 ("Ashraf *et al.*). I am also familiar with the reference entitled "Inhibitory Effect of antineoplaston A10[1] and AS2-1[2] on human hepatocellular carcinoma" by H. Tsuda, A. Iemura, M. Sata,

[1] Note that has used herein the term A10 refers to a composition comprising a mixture of phenylacetylglutamine (PAG) and phenylacetylisoglutamine (PAIG), respectively in a 4:1 ratio by mass. *Diluted* A10 refers to those compositions having a concentration of about 100 mg/mL, whereas

M. Uchida, K. Yamana, and H. Hara, *The Krume Medical* Journal, 1986, 43:137-147, ("Tsuda *et al.*"). I have thoroughly reviewed and understand the contents of both the Ashraf *et al.* and the Tsuda *et al.* references.

6. I am familiar with the distinctions of the invention disclosed and claimed in the '567 application from the materials disclosed by the Ashraf *et al.* and Tsuda *et al.* references individually and/or in combination with one another. I can state that:

(a) The compositions and methods disclosed and claimed by the '567 application exhibit a surprising and unexpected efficacy for the treatment of neoplastic disease.

(b) The compositions and methods disclosed in the '567 application are not obvious to one of ordinary skill in the art in view of the compositions and methods described and taught by the Ashraf *et al.* and Tsuda *et al.* references taken in combination.

(c) The compositions and methods disclosed and claimed by the '567 application are functionally distinct from the compositions and methods described by either the Ashraf *et al.* or the Tsuda *et al.* reference.

My conclusions are based on a thorough reading and understanding of the Ashraf *et al.* and Tsuda *et al.* references, a thorough reading and understanding of the '567 application, my experience and background in the relevant technical fields of chemistry, clinical oncology, internal medicine, physiology, pharmaceutics and oncological chemotherapy.

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concentrated A10 refers to the instantly claimed compositions having concentrations of from about 200-350 mg/mL.

7. In support of my conclusions, I have attached as Appendix A, the contents of which are hereby incorporated by reference, a report which summarizes the results of three phase II clinical studies conducted under IND (Investigational New Drug) #43,742 (these ongoing studies are supervised by the U.S. Food and Drug Administration (FDA)). All of the experimentation that is disclosed in Appendix A and hence this Declaration was either done personally by me or by individuals under my supervision and control.

8. In specific support of my conclusion and belief that the compositions and methods of the '567 patent are novel and non-obvious in view of a combination of the Ashraf *et al.* and Tsuda *et al.* references, I would like to focus the Examiner's attention on the following:

9. The Ashraf *et al.* reference teaches a formulation 100 mg/ml of Antineoplaston A10 ("A10 diluted"). The stated purpose of the study reported in this publication was to (a) study the fate of 3-phenylacetylamino-2, 6-piperidinedione when subjected to acidic and basic conditions; (b) identify the final products of hydrolysis; and (c) to perform chronic animal toxicology and stability studies of A10 injections (see page 37). Thus this publication only summarizes the results of a pre-clinical toxicity study of diluted A10 in HA/ICR Swiss white mice (see Ashraf *et al.* page 39). This study reports no administration of A10 to humans, at any concentration, nor does it not teach or suggest that the use concentrations of A10 above 100 mg/mL would be desirable.

10. The Tsuda *et al.* reference teaches that the objective of the study, described therein, was to determine the effects of "A10 and AS2-1 on cell proliferation, cell

[2] As used herein AS2-1 refers to a composition comprising a mixture of phenylacetate (PA) and phenylacetylglutamine (PAG) in a 4:1 ratio by mass.

morphology, cell cycle and DNA in human hepatocellular carcinoma cell lines" (see page 137, abstract). Pursuant to that objective the Tsuda *et al.* reference reports only (a) *in vitro* studies where in the effect of A10 and AS2-1 on six hepatocellular carcinoma cell lines was determined; and (b) a case study of one individual who took AS2-1 orally for more than 15 months. As with the Ashraf *et al.* reference, there is nothing in the Tsuda *et al.* reference which teaches or suggests that concentrated A10 would be useful. Furthermore, this reference does not report any administration of A10 to humans.

11. Clinical studies have been conducted using: (a) diluted A10[3] Burzynski an Kubove (*Drug Exptl. Clin. Res.* 1986, **12** (suppl. 1)47-55:and AS2-1); (b) diluted A10 and AS2-1[3] Buckner *et al.* (*Mayo Clin. Proc.* 1999, **75**:137-145 and (c) phenylacetate[3] Thibault *et al.* (*Cancer*, 1995, **75**:2932-2938) and Chang *et al.* (*J. Clin. Oncol.*, 1999, **17**:984-990), However, as summarized herein (see Fig. 1 of Appendix A), none of these trials have demonstrated that the respective formulations studied were efficacious within the parameters defined by FDA standards. According to FDA approved protocols new treatments should produce at least 11% "objective responses" in order to be considered active enough to merit more extensive investigation (see section 12.1 "BIOSTATISTICAL CONSIDERATIONS" in the National Cancer Institute protocol, which is included as part of Appendix B, herein). According to the National Cancer Institute and the Food and Drug Administration standards: "*objective response*" is defined as the sum of complete and partial responses. "*Complete response*" is defined as the complete disappearance of all contrast enhanced tumors on imaging studies (MRI or

[3] These references were all cited with an information disclosure statement previously submitted to the Patent Office in this application.

CT) for four weeks or longer. "*Partial response*" is defined as a greater than 50% reduction in the sum of the products of the greatest perpendicular diameters of contrast enhancing tumors for at least four weeks and no appearance of new tumors. Neither a stable disease state nor a mixed response (decrease in the size of some tumors with the concomitant increase in size of other tumors or the appearance of new tumors) will qualify as objective responses under the FDA requirements.

12. The Burzynski, and Kubove, reference describes the administration of dilute A10 to 18 cancer patients diagnosed with a variety of neoplastic diseases (see page 48 of that reference). The diluted A10 was administered as a formulation comprising 100 mg/mL given intravenously every three to six hours. An "objective response", within the FDA standards set forth above, was observed in only one of the 18 patients (see page 49).

13. The Buckner *et al.* reference describes the treatment of 9 patients with alternating administrations of diluted A10 (dosage up to 1.0 g/kg/day) and AS2-1 (dosage up to 0.4 g/kg/day). No objective response was detected for any of these patients.

14. Other studies, by Chang *et al.* and Thibault *et al.,* have investigated the antineoplastic effect of phenylacetic acid (which is both a component of AS2-1 and a breakdown product of A10). In the Chang *et al.* study, 43 patients with recurrent malignant glioma were treated with phenyacetate by continuous intravenous infusion at a rate of 400 mg/kg/day. Of those 43 patients only three (7.5%) had a response corresponding to a more than 50% reduction in tumor load (see pages 984-985). In the Thibault *et al.* study, 18 cancer patients were treated twice daily with phenylacetate at doses of between 125 mg/kg and 150 mg/kg. Of the 18 patients, only one had an objective response within the FDA definitions (see page 2932).

15. The results reported for the clinical use of A10, AS2-1, and phenylacetate, which are described above and summarized in Appendix A, herein, offer no indication that A10 and AS2-1, either alone or in combination, provide an efficacious composition for the treatment of neoplastic diseases. Analysis of the cited references and the summary of the reported results clearly shows that the use of either diluted A10 alone or in combination with AS2-1, has not been shown to provide effective treatment of neoplastic disease within the guidelines defined by FDA requirements.

16. In contrast, as reported herein, clinical trials carried out using the compositions (concentrated A10 (300 mg/mL) and AS2-1 (80 mg/mL)) and methods taught and claimed in the '567 application, resulted in objective responses of 48.6%, CAN-01 study, 54.6%, BT-09 study, and 62.5%, BT-13 study (Appendix A, herein, contains a detailed description of the dosages and protocols used in each study). This response rate is, on average, 10 times higher than that observed for the dilute A10 compositions. In each of these instantly described studies all patients were treated with daily injections of concentrated A10 (300 mg/mL) and AS2-1 (80 mg/mL).

17. Therefore, despite what is taught by a combination of the Ashraf *et al.* and Tsuda *et al.* references, the results described herein and summarized in Appendix A provide clear and convincing evidence that the compositions and methods described and claimed in the '567 application produce unexpected and surprisingly high objective response levels.

18. Upon consideration of the above, it is my conclusion that one of ordinary skill in the art would be convinced that the compositions and methods taught and claimed in the '567 application demonstrate a surprising and unexpected efficacy for the treatment of

neoplastic disease. There is nothing taught in the cited art which suggests that concentrated A10 would provide these surprisingly superior results.

19. Furthermore, although the data described and summarized is from patients treated with a combination of concentrated A10 and AS2-1 (not concentrated A10 alone), it is logical to conclude that the concentrated A10 is the agent responsible for the surprisingly good patient outcomes. As summarized in Figure I. of Appendix A, herein, AS2-1 alone has not been shown to be efficacious in the treatment of neoplastic diseases. Similarly, phenylacetate (a component of AS2-1) has been shown to be only slightly more effective (with a 5.5% and 7.5 % objective responses demonstrated in the Thibault *et al.* and the Chang *et al.* studies respectively). Significantly, as discussed above, the Buckner *et al.* study, which tested a combination of both dilute A10 and AS2-1 also failed to identify any objective response. Therefore, it my conclusion that the surprisingly high percentage of objective responses reported in the present showing must be attributed to the use of concentrated A10.

20. Consequently, I conclude that the presently claimed invention is both novel an unobvious over all previous art. More specifically, I conclude that an ordinarily skilled artisan would be convinced that the presently claimed invention is both novel and unobvious with respect to the teachings of Ashraf *et al.* and Tsuda *et. al.,* taken either separately or in combination.

21. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under section 1001 of Title

18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

August 25, 2000

Date

Stanislaw R. Burzynski

APPENDIX A

Abstracts of Phase II Studies supervised by the FDA, which demonstrate the surprisingly beneficial results achieved with the present invention and the superiority of these results with respect to the methods disclosed by the Ashraf et al., Tsuda et al. and other references . IND 43,742

S.R. Burzynski, V. Alapati, T. Janicki, G. Jurida, M. Khan, E. Kubove, J. Paszkowiak, B. Szymkowski

CAN-01 Phase II Study of Antineoplastons A10 and AS2-1 in Patients with Refractory Malignancies

Patients and Methods: This study involved patients who had failed established therapies. Patients received daily intravenous injections of antineoplastons A10, 300 mg/mL and AS2-1, 80 mg/mL at average dosages of 7.84 and 0.37 g/kg/day, respectively.

Results: Antineoplastons A10 and AS2-1 eliminated or substantially reduced tumors in 48.6% of patients with brain tumors. Of the 35 evaluable patients, nine (9) had a complete response, eight (8) a partial response, and eleven (11) a stable disease. Progressive disease occurred in seven (7) patients. 15 patients are alive today for over 4 years from the beginning of treatment. Complete and partial responses were documented in glioblastoma multiforme, anaplastic astrocytoma, astrocytoma low grade, anaplastic oligodendroglioma, oligodendroglioma, mixed glioma, medulloblastoma, and malignant meningioma.

Conclusion: Antineoplaston therapy produced complete or partial responses in 15 of 35 (48.6%) patients with brain tumors. Compared with standard treatment, antineoplaston therapy is associated with prolonged survival time and prolonged time to disease progression. Tumor dimensions were documented by magnetic resonance imaging (MRI). Changes in tumor size were categorized as defined by the National Cancer Institute.

BT-09 Phase II Study of Antineoplastons A10 and AS2-1 in Brain Tumors

Patients and Methods: Patients received daily intravenous injections of antineoplastons A10, 300 mg/mL and AS2-1, 80 mg/mL at average dosages of 8.19 g/kg/day and 0.33 g/kg/day, respectively.

Results: Antineoplastons A10 and AS2-1 eliminated or substantially reduced tumors in 54.5% of patients with brain tumors. Of the 11 evaluable patients, one (1) had a complete response, five (5) a partial response, and four (4) a stable disease.

Progressive disease occurred in one patient. Complete and partial responses were documented in astrocytoma, medulloblastoma and glioma

Conclusion: Antineoplaston therapy produced complete or partial responses in 6 of 11 (54.5%) patients with brain tumors. Tumor dimensions were documented by magnetic resonance imaging (MRI). Changes in tumor size were categorized as defined by the National Cancer Institute.

BT-13 Phase II Study of Antineoplaston A10 and AS2-1 in Children with Low Grade Astrocytoma.

Patients and Methods: Patients received daily intravenous injections of antineoplastons A10, 300 mg/mL and AS2-1, 80 mg/mL at average dosages of 15.99 g/kg/day and 0.59 g/kg/day, respectively.

Results: Antineoplastons A10 and AS2-1 eliminated or substantially reduced tumors in 62.5% of patients with brain tumors. Of the 8 evaluable patients, two (2) had a complete response, three (3)a partial response, and two (2) a stable disease. Progressive disease occurred in one case. Complete and partial responses were documented in astrocytoma low grade.

Conclusion: Antineoplaston therapy produced complete or partial responses in 5 of 8 (62.5%) patients with brain tumors. Tumor dimensions were documented by magnetic resonance imaging (MRI). Changes in tumor size were categorized as defined by the National Cancer Institute.

Table I

Tabulation of Data which Demonstrates the Surprisingly Beneficial Results Achieved with the Present Inventions and the Superiority of these Results with Respect to the Methods Disclosed by the Ashraf et al., Tsuda et al. and other References

	Study	Drugs	Number of Evaluable Cases	Objective Response Number of Patients	Objective Response % of Patients	Efficacy by FDA's Standards
Cited Art	Ashraf et al. [1)]	A10 diluted	None	None	None	None
	Tsuda et al. [2)]	AS2-1	1	None	None	None
	Burzynski and Kubove [3)]	A10 diluted	18	1	5.5	None
	Buckner et al. [4)]	AS2-1 and A10 diluted	6	None	None	None
	Thibault et al. [5)]	Phenylocetate	18	1	5.5	None
	Chang et al. [6)]	Phenylocetate	40	3	7.5	None
Present Invention	Burzynski et al. [7)]	AS2-1 and A10 concentrated	35	17	48.6	Effective
	Burzynski et al. BT-09 Study	AS2-1 and A10 concentrated	11	6	54.6	Effective
	Burzynski et al. BT-13 Study	AS2-1 and A10 concentrated	8	5	62.5	Effective

References:

1) Ashraf, A. Q.; Liau, M. C.; Mohabbat, M. O.; Burzynski, S. R. Preclinical studies on Antineoplaston A10 injection. Burzynski Res. Inst., Inc., Stafford, Tx. 7477, USA Drugs Exp. Clin. Res. 1986; 12 (suppl. 1), 37-45.

2) Tsuda, Hideaki; Iemura, Akihiro; Sata, Michio; Uchida, Masafumi; Yamana, Kazunari; Hara, Hiroshi. Inhibitory effect of Antineoplastons A10 and AS2-1 on human hepatocellular carcinoma. Dep. Anesthesiology, Kurum Univ. Sch. Med., Kurume, 830, Japan Kurume Med. J. 1996; 43(2), 137-147.

3) Burzynski, S.R., Kubove, E. Toxicology studies of Antineoplaston A10 injections in cancer patients. Drugs Exptl Clin Res. 1986; 12 (suppl 1): 47-55.

4) Buckner , J. C., Malkin, M. G., Reed, E., Cascino, T. L. Reid, J. M., Ames, M. M., Tong, W. P., Lim, S., Figg, W. D. Phase II Study of Antineoplastons A10 (NSC 648539) and AS2-1 (NSC 620261) in Patients with Reccurent Glioma. Mayo Clin Pro. 1999; 74: 137-145.

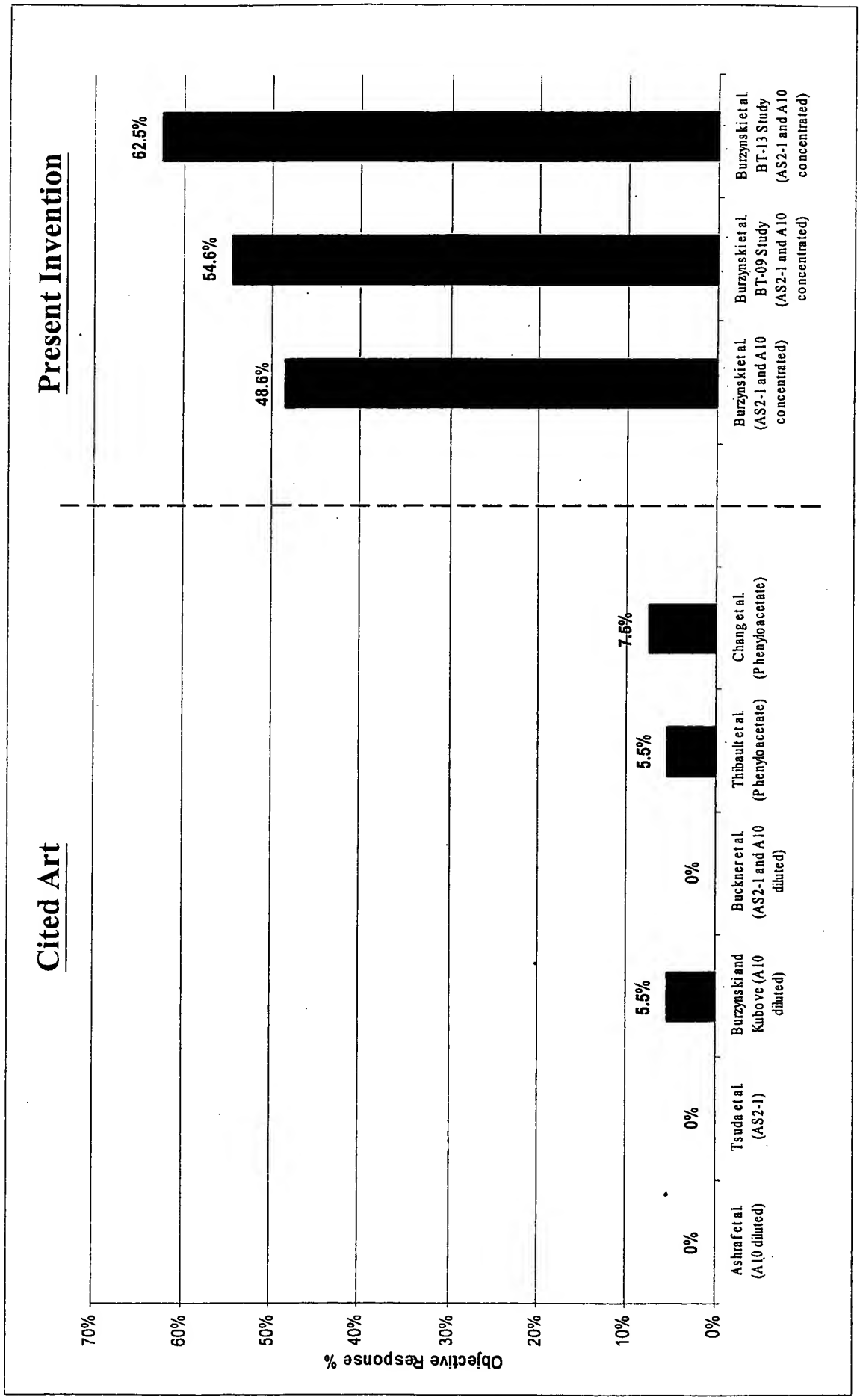
5) Thibault et al, "Phase I Study of Phenylacetate Administered Twice Daily to Patients with Cancer", Cancer, Vol. 75, 1995; 2932-2938.

6) Chang et al, "Phase II Study of Phenylacetate in Patients with Reccurent Malignant Glioma: A North American Brain Tumor Consortium Report", Journal of Clinical Oncology, Vol. 17, No. 3 March 1999; 984-990.

7) Burzynski , S. R., Conde, Arthur B., Peters, Alonzo, Saling, Benjamin, Ellithorpe , Rita, Daugherty, James P., and Nacht, Carl H. A Retrospective Study of Antineoplastons A10 and AS2-1 in Primary Brain Tumors. Clin Drug Invest Jul: 18(1): 1-10. This Publication presents 36 evaluable patients, 16 of these patients reached objective response which was 44.4 %. Since this publication the data have been updated.

Fig. 1

Graph of Data which Demonstrates the Surprisingly Beneficial Results Achieved with the Present Inventions and the Superiority of these Results with Respect to the Methods Disclosed by the Ashraf et al., Tsuda et al. and other References



APPENDIX B

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

COPY

EPN-718
(301)496-7912

DATE: April 8, 1994

TO: Stanislaw R. Burzynski, M.D., Ph.D.
Burzynski Research Institute, Inc.
12000 Richmond, Suite 260
Houston, TX 77082-2431

FROM: Regulatory Affairs Branch, Cancer Therapy Evaluation Program, DCT, NCI

SUBJ: ANTINEOPLASTON

The following has recently been forwarded to the Food and Drug Administration by the Division of Cancer Treatment, NCI. A copy of each item is enclosed.

Protocol #:	Description:	Serial No.
T93-0078	Amendment	010
T93-0143	New Protocol	011

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
INVESTIGATIONAL NEW DRUG APPLICATION (IND)
(TITLE 21, CODE OF FEDERAL REGULATIONS (CFR) Part 312)

Form Approved: OMB No. 0910-0014.
Expiration Date: November 30, 1990.
See CMB Statement on Reverse.

NOTE: No drug may be shipped or clinical investigation begun until an IND for that investigation is in effect (21 CFR 312.40).

1. NAME OF SPONSOR
DIVISION OF CANCER TREATMENT, NCI

2. DATE OF SUBMISSION
March 10, 1994

3. ADDRESS *(Number, Street, City, State and Zip Code)*
National Institutes of Health
Executive Plaza North, Room 718
Bethesda, Maryland 20892

4. TELEPHONE NUMBER *(Include Area Code)*
(301) 496-7912

5. NAME(S) OF DRUG *(Include all available names: Trade, Generic, Chemical, Code)*
ANTINEOPLASTON AS2-1

6. IND NUMBER *(If previously assigned)*
43,013

7. INDICATION(S) *(Covered by this submission)*

8. PHASE (S) OF CLINICAL INVESTIGATION TO BE CONDUCTED: ☐ PHASE 1 ☐ PHASE 2 ☐ PHASE 3 ☐ OTHER _____ *(Specify)*

9. LIST NUMBERS OF ALL INVESTIGATIONAL NEW DRUG APPLICATIONS *(21 CFR Part 312)*, NEW DRUG OR ANTIBIOTIC APPLICATIONS *(21 CFR Part 314)*, DRUG MASTER FILES *(21 CFR 314.420)*, AND PRODUCT LICENSE APPLICATIONS *(21 CFR Part 601)* REFERRED TO IN THIS APPLICATION.

10. *IND submissions should be consecutively numbered. The initial IND should be numbered "Serial Number: 000." The next submission (e.g., amendment, report, or correspondence) should be numbered "Serial Number: 001." Subsequent submissions should be numbered consecutively in the order in which they are submitted.*

SERIAL NUMBER: 0 1 0

11. THIS SUBMISSION CONTAINS THE FOLLOWING: *(Check all that apply)*

☐ INITIAL INVESTIGATIONAL NEW DRUG APPLICATION (IND)
☐ RESPONSE TO CLINICAL HOLD

PROTOCOL AMENDMENT(S):
- ☐ NEW PROTOCOL
- ☒ CHANGE IN PROTOCOL
- ☐ NEW INVESTIGATOR

INFORMATION AMENDMENT(S):
- ☐ CHEMISTRY/MICROBIOLOGY
- ☐ PHARMACOLOGY/TOXICOLOGY
- ☐ CLINICAL

IND SAFETY REPORT(S):
- ☐ INITIAL WRITTEN REPORT
- ☐ FOLLOW-UP TO A WRITTEN REPORT

☐ RESPONSE TO FDA REQUEST FOR INFORMATION
☐ ANNUAL REPORT
☐ GENERAL CORRESPONDENCE

☐ REQUEST FOR REINSTATEMENT OF IND THAT IS WITHDRAWN, INACTIVATED, TERMINATED OR DISCONTINUED
☐ OTHER _____ *(Specify)*

CHECK ONLY IF APPLICABLE

JUSTIFICATION STATEMENT MUST BE SUBMITTED WITH APPLICATION FOR ANY CHECKED BELOW. REFER TO THE CITED CFR SECTION FOR FURTHER INFORMATION.

☐ TREATMENT IND 21 CFR 312.35(b) ☐ TREATMENT PROTOCOL 21 CFR 312.35(a) ☐ CHARGE REQUEST/NOTIFICATION 21 CFR 312.7(d)

FOR FDA USE ONLY

CDR/DBIND/DGD RECEIPT STAMP	DDR RECEIPT STAMP	IND NUMBER ASSIGNED:
		DIVISION ASSIGNMENT:

FORM FDA 1571 (7/90) PREVIOUS EDITION IS OBSOLETE.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892

Executive Plaza North
Room 718
(301) 496-7912

Date: 03/11/94

NSC: 620261
IND: 43013
ANTINEOPLASTON AS2-1
AMEND #: 1
FILED: 11/26/93

NSC: 648539
IND: 43013
ANTINEOPLASTON A10
AMEND #: 1
FILED: 11/26/93

Gregory M. Burke, M.D., Ph.D.
Ctr Drug Eval & Research, FDA
Oncology Group, HFD-150
ATTN: Document Room Suite 200 North
1401 Rockville Pike
Rockville, MD 20852

Dear Dr. Burke:

Enclosed are three copies of a protocol amendment for T93-0078, "PHASE II STUDY OF ANTINEOPLASTONS A10 AND AS2-1 IN PATIENTS WITH ADVANCED RECURRENT MALIGNANT ASTROCYTOMAS", which are submitted as an amendment to Item 6 of the above referenced INDs .

Division of Cancer Treatment, NCI
(Sponsor)

Sincerely yours,

Dale Shoemaker, Ph.D.
Chief, Regulatory Affairs Branch
Cancer Therapy Evaluation Program

Enclosure

JAN 26 1994

January 24, 1994

Mario Sznol, M.D.
Investigational Drug Branch
CTEP, DCT, NCI
6130 Executive Boulevard
Executive Plaza North, Room 715
Rockville, Maryland 20852

Dear Dr. Sznol:

In follow-up of our previous correspondence, I resubmit an amended version of NCI protocol #: T-93-0078, entitled "**Phase II Study of Antineoplastons A10 and AS2-1 in Patients with Advanced Recurrent Malignant Gliomas**". Based upon the extended conference call with you, and Investigators from the Mayo Clinic and NCI's Clinical Pharmacology Branch, the following changes were made:

1. As indicated on the Face Page, the Clinical Pharmacology Branch at NCI has been added as a 3rd participating Center.

2. Subsections "l)" and "m)" have been added to Section 1.1 refining the eligibility requirements further; a parallel change has been made in 6.1, l) and 6.2, j).

3. Sections 1.3 and 5.1 have been amended to specify that the length of the programmed individual twice hourly alternating injections of the two antineoplaston preparations will be 15 minutes.

4. The pharmacokinetic studies sections (1.4 and 5.3) have been amended with respect to the days and times of the sampling. With the modified sampling times the total blood volume required for pharmacokinetic studies has been increased from 120 ml to 150 ml; this required a change in the Consent Form (Page 2 last paragraph).

5. In Sections 1.5 and 8.5 the follow up intervals for MRI or CT scans were modified to reconcile the timing with that of the actual clinical follow up assessment. It increases the frequency of assessment slightly from the third year onward.

Sincerely,

Mark Malkin, M.D.

xc Jerome Posner, M.D., Charles Young, M.D., John Lewis, M.D.

Proposal for Clinical Investigation

PHASE II STUDY OF ANTINEOPLASTONS A10 AND AS2-1 IN PATIENTS WITH ADVANCED RECURRENT MALIGNANT ASTROCYTOMAS*

N.C.I. Protocol # T93-0078

Protocol Chairman, and Coordinating Center

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Institutional Principal Investigators

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Co-Investigators

[MSKCC] Jerome Posner, M.D., Frank Lieberman, M.D., Charles Young, M.D., William Tong, Ph.D.

[Mayo Clinic] Joel Reid, Ph.D., Randall Millikan, M.D.

[NCI] William D. Figg, Pharm.D.

Department of Neurology, Developmental Chemotherapy Service, Department of Medicine, Core Pharmacology Laboratory, Memorial Sloan-Kettering Cancer Center

Department of Medical Oncology, Mayo Clinic

Clinical Pharmacology Branch, National Cancer Institute

* Antineoplaston A10 is NSC 648539; Antineoplaston AS2-1 is NSC 620261

1.0 Protocol Summary - Phase II Study of Antineoplastons A10 and AS2-1 in Patients with Advanced Recurrent Malignant Astrocytomas

1.1 Patient Eligibility - a) Histologically or cytologically confirmed diagnosis of malignant astrocytoma, reviewed at MSKCC, progressed during or recurrent following initial therapy, including radiation therapy. b) PS - Karnofsky 100 to 70%, ECOG 0 to 1; life expectancy $\geq$ 4 months. c) Hgb $\geq$ 10 gm/dL, WBC > 2000, granulocytes > 1500, Plt > 100,000, Creatinine $\leq$ 2.0 mg/dl, bilirubin $\leq$ 2.0 mg/dl. d) Informed consent by a patient who is competent to provide it, or by their legal guardian in case of tumor-induced aphasia. e) Geographic location adequate for follow-up. f) No radiation therapy for at least 8 weeks, no chemotherapy for at least 4 weeks prior to starting the protocol (6 weeks for nitrosoureas). Patients must have recovered from the myelosuppressive effects associated with any prior chemotherapy or radiation therapy. g) $\geq$ 18 years of age. h) Patients with a malignancy other than *in situ* carcinoma of the cervix are excluded. i) Patients with known heart disease of New York Heart Association Class III - IV are excluded, as are patients with any history of congestive heart failure, symptomatic coronary vascular disease (as manifest by a myocardial infarction within the previous year or angina requiring medication), or moderate to severe chronic obstructive pulmonary disease. j) Patients who are pregnant or breast feeding are excluded. k) Patients with tumors > 5 cm in diameter, multifocal tumors, or with leptomeningeal or systemic metastases are excluded. l) Patients must be on a fixed dose of corticosteroids or on no corticosteroids for at least one week prior to the baseline scan. m) No prior or concurrent therapy with antineoplastons, phenylacetic acid or phenylbutyric acid.

1.2 Pretreatment Evaluation - a) Complete history and physical examination, including neurologic examination. b) Height, weight and performance status. c) complete blood count and differential, PT, APTT, biochemical screening profile, creatinine, electrolytes, glucose, blood ammonia, baseline levels of anti-epileptic agents, glutamine, and glutamate in the context of the Pharmacology studies. e) Chest x-ray, PA and lateral. f) EKG. g) baseline contrast-CT and/or MRI scan with gadolinium contrast for known areas of tumor involvement. h) Urinalysis.

1.3 Treatment Plan - Treatment will begin within 7 days of the baseline neuroimaging scan. A double lumen Broviac, Groshung or equivalent catheter will be necessary for treatment. The two Antineoplastons are to be administered through separate lumens. Patients will receive gradually escalating doses of the two antineoplastons by multiple intermittent intravenous injections (twice hourly) using a portable programmable pump. The initial day's dosage will be 0.24 g/kg/day for Antineoplaston A10 and 0.12 g/kg/day for Antineoplaston AS2-1; with each succeeding day there will be an escalation of the dose of Antineoplaston A10 by 0.24 g/kg/day and Antineoplaston AS2-1 by 0.12 g/kg/day, as tolerated, until target doses are reached of 1 gram/kg/day of Antineoplaston A10 and 0.4 grams/kg/day of Antineoplaston AS2-1. For a 70 kg patient the daily dosages would be 70 grams of Antineoplaston A10 and 28 grams of Antineoplaston AS2-1. Since the average drug concentration being administered is 80 mg/ml the projected infusate volume/day is 1225 ml in a 70 kg patient. The length of the programmed individual injections will be 15 minutes. Dose adjustments will be made in the event of toxicity; a detailed schedule is provided in Section 5.2.

The initial week of therapy will be undertaken in the hospital in order to assess the patient's tolerance to therapy, to measure the pharmacokinetic parameters for the intermittent injections, and to train the family in the use of the pump and replacement of the infusion bags. Subsequent administration will be on an Outpatient basis, supported by the Center and visits by nurses from a home infusion support company. Duration of

Therapy: Therapy will continue indefinitely until the development of progressive disease, unacceptable toxicity or intervening medical problems. Patients achieving CR will continue for 8 months beyond the date of CR. Patients with stable disease or a PR may be treated until the disease progresses.

1.4 Pharmacokinetic studies will be carried out in all patients on Days 1, 3, 5, 8, and 15. A green topped tube will be drawn for analysis of phenylacetic acid (PAA), phenylacetylglutamine (PAG) and phenylacetylisoglutamine at times: 0 = baseline, 15, and 30 minutes, timing the samples just prior to the initiation of sequential pulsed injections of Antineoplastons A10, AS2-1, and A10 on the given day. Total blood drawn for the initial pharmacokinetic studies: 150 cc. Monthly blood samples will also be obtained on an Outpatient basis for PAA and PAG to assess the reproducibility of long-term levels of the drug. Urine samples will be analyzed for PAA and PAG; samples will be obtained: 24 hour pre-collection, 0-24, 48-72 and 96-120 hours.

1.5 Evaluation During Study - History and physical examination, including neurological examination every four weeks for two years, eight weeks for the 3rd year, CBC, Screening Profile, electrolytes, serum anti-epileptic levels Days 1, 4, 8, 15, 29 and every four weeks thereafter. Follow up MRI or CT scans at 8 week intervals for three years, at 12 week intervals for the fourth year, at 16 week intervals for the fifth year, 24 week intervals for the sixth year, then yearly.

1.6 Possible side effects of therapy - Based on previous clinical studies, Antineoplaston therapy can produce the following toxic effects: dizziness, nausea and vomiting; however, the following are considered to be possible additional side effects: anemia, leukopenia, thrombocytopenia, hypocalcemia, hypertension, metabolic acidosis, fluid overload (edema), hypoglycemia, hypokalemia, weakness, fever, myalgias, and abdominal pain. Drug administration requires the use of an indwelling central venous catheter; insertion of this device can be accompanied by local discomfort, pneumothorax, hemothorax, and subclavian artery puncture. Prolonged drug administration by way of the central venous line, can produce infection of that line and phlebitis of the infused blood vessel.

2.0 OBJECTIVES

2.1 To determine the objective response rate in patients with advanced malignant astrocytomas to combined therapy with Antineoplastons A10 1 g/kg/day and AS2-1 0.4 g/kg/day administered by repeated pulsed intravenous injections twice hourly indefinitely until the development of progressive disease.

2.2 To describe the patients' tolerance to and the side effects of this regimen.

2.3 To describe the pharmacokinetic behavior and metabolism of Antineoplastons A10 and AS2-1 in regard to the plasma content of PAA, PAG and phenylacetylisoglutamine (PAisoG) at steady state, and the urinary content and cumulative excretion of these compounds.

3.0 BACKGROUND INFORMATION

3.1 Malignant gliomas, which affect approximately 13,000 adults each year in the United States, remain incurable primary brain tumors despite surgical resection, radiation therapy and chemotherapy. The median survival for glioblastoma multiforme, the most

common and most aggressive primary central nervous system neoplasm, is roughly 12 months. Younger age, lower histopathologic grade, higher postoperative performance status, more complete extent of resection, and a tumor dose of more than 5,000 cGy confer a better prognosis, but the fact remains that fewer than 5% of patients with malignant glioma will survive more than 5 years from diagnosis. Refinements in neuroanesthesia, neurosurgical technique, radiation therapy (including interstitial brachytherapy and stereotactic radiosurgery), and conventional chemotherapy (either single or multiple agents) are unlikely to provide significant improvement in tumor control or increase the likelihood of long-term survival. In view of the unfavorable survival outlook with currently available treatment modalities, it is appropriate to evaluate unusual agents that appear to produce clinical antitumor effects in patients with malignant glioma.

3.2 Antineoplastons are chemically defined compounds that were originally isolated from urine and are now produced using large scale synthetic methods. The National Cancer Institute has recently reviewed the charts, MRI/CT scans and pathology slides of seven patients with primary brain tumors who were treated with a combination of antineoplastons A10 (3-(N-phenyl-acetamino)-2,6-piperidinedione), which forms phenylacetylglutamine and phenylacetylisoglutamine in a 4:1 ratio upon hydrolysis) and AS2-1 (phenylacetylglutamine and phenylacetic acid in a 1:4 ratio upon hydrolysis). These cases represented a selected group of patients (a so-called 'best case series') prepared by Dr. S. R. Burzynski for the NCI review. In the NCI review of the selected case materials there appeared to be an association between antineoplaston administration and objective tumor regressions (described below).[1] Based upon that review, and responsive to a Congressional mandate to "test the most promising unconventional medical practices", NCI has elected to sponsor four extramural Phase II trials of Antineoplastons A10 and AS2-1 in patients with malignant glioma, the drugs being administered on an outpatient basis by continuous intravenous infusion using programmable pumps.

3.3 Results of the NCI Review:[1] In two adult patients with glioblastoma multiforme there was 1 CR which lasted 6 months and 1 MR of short duration. Of two adult patients with anaplastic astrocytomas, one patient with a brain stem primary achieved a CR which is ongoing for 3+ years (1 year after completing therapy); the other patient had calcification of her primary tumor. The other adult patient had a histologically confirmed Grade 3 astrocytoma with intracerebral metastases (not biopsied) which resolved completely while the primary lesion decreased by over 50%. At the time of review this patient had received antineoplastons for over 3 years, after having previously failed XRT, procarbazine, CCNU, and vincristine, interferon-Beta and MGBG/DFMO. All of the adult cases had previously received radiotherapy. Two pediatric cases were reviewed; a child with a Grade 1 astrocytoma achieved a PR for 5+ months; a child with an anaplastic astrocytoma achieved a CR for 9+ months. Although there has been no peer-reviewed publication of antineoplaston therapy in brain tumors, Burzynski et al have reported 6 objective responses and 10 disease stabilization in 20 patients with advanced astrocytomas treated with a range of doses of Antineoplastons A10 and AS2-1.[2]

3.4 One of the major hydrolysis products of Antineoplaston AS2-1 is phenylacetic acid (PAA); this compound has established biologic activity. PAA and its prodrug phenylbutyric acid have been successfully used for over two decades in the treatment of children who are hyperammonemic because of genetic defects in urea metabolism.[3-5] Furthermore, Samid and her colleagues have demonstrated that PAA is an active cytodifferentiating agent in cell culture systems at media concentrations (5 mM) typical of the peak levels occurring in children who receive PAA intravenously as therapy for hyperammonemia.[6] The spectrum of cells demonstrating cytodifferentiation in the presence of PAA has included HL-60 and K-562 leukemia, and preadipocytes; differentiation in HL-60 was associated

with a rapid decline in *myc* oncogene expression. PAA has also been growth inhibitory to human neoplastic cell lines of lung, prostate, melanoma, and glial tumor origin; the PAA-treated cells lost their capacity to proliferate in agar culture.[6]

3.5 PAA lowers ammonia levels through its mitochondrial conjugation with glutamine, generating phenylacetylglutamine (PAG), which is then excreted in the urine; in this process plasma glutamine levels are lowered also.[3,4] PAG, the principal hydrolysis product of Antineoplaston A10, was inactive in Samid's cytodifferentiation and growth inhibition studies.[6]

3.6 Tolerance of Antineoplaston Therapy - Burzynski has reported negligible toxicity from the combined use of Antineoplaston A10 0.8 to 1.2 g/kg/day and AS2-1 0.2-0.5 g/kg/day.[2] Evidently this was in accord with the findings of the NCI panel that reviewed the records of the test cases.[1] The experience with PAA is more extensive; total daily doses of up to .5 g/kg/day (20 g/m^2) are well tolerated indefinitely.[7] Since the intravenous Antineoplaston preparations will be administered as the sodium salt, patients should be followed closely for development of edema or other signs of sodium overload.

3.7 Pharmacokinetic behavior of Antineoplastons - Although there are no available data concerning the clinical pharmacokinetic behavior of the antineoplastons, PAA is reported to have a first order decay with a half-life of 254 minutes in humans following intravenous infusion of 12 g/m^2 (.3 g/kg) over 90 minutes.[5] With the combined administration of Antineoplastons A10 and AS2-1 it is likely that treated patients are receiving both phenylacetic acid and phenylacetylglutamine, the end product of the catabolism of PAA. If the PAA --> PAG conjugation is subject to end product inhibition, it is possible that the result would be a more prolonged plasma and tissue half-life for PAA.

4.0 PHARMACEUTICAL INFORMATION

4.1 Antineoplaston A10 (NSC 648539) and Antineoplaston AS2-1 (NSC 620261) are investigational agents supplied by the Burzynski Research Institute, Inc. to the Division of Cancer Treatment, NCI for clinical trials. Chemically synthesized Antineoplaston A10 is identified by its NMR and infrared characteristics as 3-(N-phenyl-acetamino)-2,6-piperidinedione. In aqueous solution this hydrolyzes to phenylacetylglutamine and phenylacetylisoglutamine in a 4:1 ratio. Antineoplaston A10 infusion is a sterile solution of phenylacetyl glutamine and phenylacetylisoglutamine in water which has the pH adjusted to 7.0 with sodium hydroxide. Each ml of the infusion contains not less than 62 mg and not more than 66 mg of phenylacetylglutamine and not less than 14 mg and not more than 18 mg of phenylacetylisoglutamine. The combined concentration of active ingredients is 80 $\pm$ 4 mg/ml.

Antineoplaston AS2-1 infusion is a sterile solution of phenylacetic acid and phenylacetylglutamine in water which has pH adjusted to 7.0 with sodium hydroxide. Each ml of the infusion contains not less than 62 mg and not more than 66 mg of phenylacetic acid and not less than 15 mg and not more than 17 mg of phenylacetylglutamine. The combined concentration of active ingredients is 80 $\pm$ 3 mg/ml.

Antineoplaston A10 and AS2-1 infusions are available in 100 ml multiple dose containers of Type I glass for single dose infusion.

4.2 Storage: Antineoplastons A10 and AS2-1 are stored at room temperature (15-30°C) without refrigeration or freezing.

4.3 Stability: Antineoplaston A10 and AS2-1 infusions are stable for 24 months.

5.0 TREATMENT PLAN AND PHARMACOKINETIC STUDIES

5.1 Treatment will begin within 7 days of the baseline neuroimaging scan. A double lumen Broviac, Groshung or equivalent catheter will be necessary for treatment. The two Antineoplastons are to be administered through separate lumens. Patients will receive gradually escalating doses of the two antineoplastons by multiple intermittent intravenous injections (twice hourly) using a portable programmable pump. The initial day's dosage will be 0.24 g/kg/day for Antineoplaston A10 and 0.12 g/kg/day for Antineoplaston AS2-1; with each succeeding day there will be an escalation of the dose of Antineoplaston A10 by 0.24 g/kg/day and Antineoplaston AS2-1 by 0.12 g/kg/day, as tolerated, until target doses are reached of 1 gram/kg/day of Antineoplaston A10 and 0.4 grams/kg/day of Antineoplaston AS2-1. For a 70 kg patient the daily dosages would be 70 grams of Antineoplaston A10 and 28 grams of Antineoplaston AS2-1. Since the average drug concentration being administered is 80 mg/ml the projected infusate volume/day is 1225 ml in a 70 kg patient. The length of the programmed individual injections will be 15 minutes, varying the flow rate depending on the volume to be infused. In the event that any adverse event occurs, the infusion will be stopped and the principal investigator will be notified immediately; reinstitution of the infusion will be at the discretion of the principal investigator. The initial week of therapy will be undertaken in the hospital in order to assess the patient's tolerance to therapy, to measure the pharmacokinetic parameters for the intermittent injections, and to train the family in the use of the pump and replacement of the infusion bags. Subsequent administration will be on an Outpatient basis, supported by the Center and visits by nurses from a home infusion support company. The infusion bags containing the Antineoplastons A10 and AS2-1 are to be replaced every 24 hours in order to minimize the risk of bacterial contamination. The infusion bags of Antineoplastons A10 and AS2-1 should be administered to the patients within 24 hours of preparation.

5.2 Dose attenuation schedule - Dose adjustments in relation to patient tolerance will be as follows:

Full dosage: Granulocytes > 1,500, platelets > 100,000.

Decrease the dosage by 25%: Increased sleepiness and weakness.

Decrease the dosage by 50%: Granulocytes < 1,000 but > 500, platelets < 100,000 but > 50,000, Patient morbidity such as nausea, vomiting.

Discontinue therapy: Platelets < 50,000, Allergic skin reactions. In the event of marked elevation of transaminases and total bilirubin therapy should be discontinued for two days and restarted at 50% of the previous dosage. It may then be reescalated to full dosage if the liver functions progressively return toward normal.

Dose adjustments not specifically covered above: For Grade 3 or 4 toxicity, the drugs will be held until that toxic effect has reduced to Grade 1 or less; therapy will then be restarted with a 25% dose reduction. If there is a recurrence of any Grade 3 or 4 toxicity at the reduced dose, the procedure will be repeated with a dose reduction to 50% of the original level. Patients should be removed from study for a third episode of Grade 3 or 4 toxicity or for any Grade 4 toxic effect that is life threatening or is not easily and rapidly reversible.

If dose-limiting toxicity develops during the dose-escalation phase of therapy, the drugs will be discontinued and restarted at the highest level that had been tolerated. It is reasonable to restart the dose-escalation if the patient is then able to tolerate therapy at that level for at least 2 days. If a second dose-limiting toxic episode occurs, the drugs will be discontinued and reinitiated, after toxicity has resolved, at the highest tolerated dose without further escalation.

Miscellaneous: Hyperuricemia ---> Administer allopurinol; Hypokalemia ---> Administer KCl.

5.3 Pharmacokinetic studies will be carried out in all patients on Days 1, 3, 5, 8, and 15. A green topped tube will be drawn for analysis of phenylacetic acid (PAA), phenylacetylglutamine (PAG) and phenylacetylisoglutamine at times: 0 = baseline, 15, and 30 minutes, timing the samples just prior to the initiation of sequential pulsed injections of Antineoplastons A10, AS2-1, and A10 on the given day. Total blood drawn for the initial pharmacokinetic studies: 150 cc. Monthly blood samples will also be obtained on an Outpatient basis for PAA and PAG to assess the reproducibility of long-term levels of the drug. Urine samples will be analyzed for PAA and PAG; samples will be obtained: 24 hour pre-collection, 0-24, 48-72 and 96-120 hours.

5.4 Duration of Therapy: Therapy will continue indefinitely until the development of progressive disease, unacceptable toxicity or intervening medical problems. Patients achieving CR will continue for 8 mo beyond the date of CR. Patients with stable disease or a PR may be treated until the disease progresses.

6.0 PATIENT ELIGIBILITY

6.1 Inclusion Criteria

a) Histologically confirmed anaplastic astrocytoma or glioblastoma multiforme that has progressed during, or is recurrent following, initial therapy, including radiation therapy. The diagnostic slides are to have been reviewed by the Neuropathologist at Memorial Hospital. Patients will be stratified for diagnosis, anaplastic astrocytoma *versus* glioblastoma multiforme at entry into the protocol, and in the subsequent analysis.

b) Radiologic evidence of recurrent or progressive tumor, following prior therapy, including radiation therapy, by gadolinium-enhanced MRI or, if MRI is contra-indicated, contrast-enhanced CT scan, within one week of study entry.

d) Eight weeks must have elapsed since the last dose of radiation therapy. Patients who have received interstitial radiation therapy, or stereotactic radiosurgery, are eligible. Four weeks must have elapsed since the last dose of chemotherapy (six weeks since the last dose if a nitrosourea), immunotherapy, or any other biological response modifier. Patients who have previously received a cytodifferentiating agent are eligible.

e) Patients may be male or female. If female, the patient must not be pregnant or breast-feeding an infant, and either incapable of becoming pregnant or currently using contraceptive methods. Acceptable methods include the birth control pill, use of a diaphragm, intrauterine device, or condom by the patient's sexual partner. If the patient is a sexually active male, the patient should take contraceptive measures to prevent possible conception with genetically damaged sperm.

f) Patient must be 18 years of age or older.

g) Patient must sign the informed consent form, indicating an awareness of the experimental nature of this study. In the event that the patient has impairment of higher intellectual function, eg. aphasia, then the patient's legal next of kin or legal guardian must sign the informed consent form indicating an awareness of the experimental nature of the study.

h) Patient must be geographically accessible for follow-up.

i) Patient must have life expectancy of four months or more. The patient's Performance Status should be consistent with Outpatient therapy, i.e. 70% to 100% Karnofsky, or 0 to 1 ECOG. The use of corticosteroids is permitted to reduce symptoms and signs attributed to cerebral edema. It is recommended that the smallest dose be used compatible with the preservation of optimal neurologic function.

j) Minimal hematological parameters include a hemoglobin of at least 10 gm/dl, a white blood count of at least 2,000, an absolute granulocyte count of at least 1500, and a platelet count of at least 100,000.

k) Evidence of adequate renal and hepatic function: Creatinine < 2.0 mg/dl, Total Bilirubin < 2.0 mg/dl.

l) Must be on a fixed dose of corticosteroids or on no corticosteroids for at least one week prior to the baseline scan.

6.2 **Exclusion Criteria**

a) Patients with tumors > 5 cm in diameter, multifocal tumors, or with leptomeningeal or systemic metastases are excluded.

b) Patient either pregnant or breast-feeding an infant.

c) Patient is a poor medical or psychiatric risk, having non-malignant systemic disease which would, in the opinion of the investigator, make therapy with an investigational drug unwise.

d) Patient is incompetent to give informed consent to treatment.

e) Presence of active infection.

f) Myelosuppressive chemotherapy or myelosuppressive radiotherapy in the past four weeks, (six weeks for nitrosourea). Patients must have recovered from the myelosuppressive effects associated with any prior chemotherapy or radiation therapy.

g) Concurrent chemotherapy.

h) Patients with malignancy other than *in situ* carcinoma of the cervix.

i) Patients with known heart disease of New York Heart Association Class III or IV. Due to the sodium load and the volume of the drug infusion, patients with any history of congestive heart failure, symptomatic coronary vascular disease (as manifest by a myocardial infarction within the previous year or angina requiring medication), or

moderate to severe chronic obstructive pulmonary disease will be excluded.

j) Previous or concurrent treatment with antineoplastons, phenylacetic acid or phenylbutyric acid.

7.0 PRETREATMENT EVALUATION

7.1 Complete history and physical examination, including neurologic examination with documentation of all signs and symptoms, height, weight, body surface area, analgesia requirement, and Performance Status.

7.2 Complete blood count with platelet count, differential, reticulocyte count, and evaluation of the peripheral smear.

7.3 Complete urinalysis.

7.4 Biochemical screening profile (BUN, uric acid, calcium, total bilirubin, total protein, albumin, inorganic phosphorous, alkaline phosphatase, SGOT, LDH, glucose) and serum creatinine. Tumor marker studies should be included where appropriate. Blood ammonia, glutamine and glutamate levels will be measured in the context of pharmacokinetic studies at time zero. Baseline serum levels of anti-epileptic agents.

7.5 Prothrombin time (PT), activated partial thromboplastin time (APTT).

7.6 EKG, Chest PA, and Lateral. Neuroimaging studies as described in Section 6.1, b).

7.7 Lesion measurements should be made by direct caliper determination when possible and, if clinically indicated, by radiographs, scintigraphs, and sonographs.

7.8 All patients will be centrally registered by telephone with the Coordinating Center [MSKCC]. The Registrar is Dr. Mark Malkin, 212-639-6688. Dr. Malkin will check the eligibility criteria for all patients as outlined in Section 6.0.

8.0 EVALUATION DURING STUDY

8.1 Detailed history and physical examination with recording of relevant symptoms/signs and Performance Status at least every four weeks for 2 years, eight weeks for the 3rd year, 12 weeks for the fourth year, sixteen weeks for the fifth year, twenty-four weeks for the sixth year, and yearly thereafter.

8.2 Historical assessment and recording of the patient's symptoms daily during the Inpatient drug administration period then every four weeks thereafter.

8.3 Complete blood count, biochemical screening profile, electrolytes and serum anti-epileptic levels on Days 1, 4, 8, 15, and 29, and every four weeks thereafter.

8.4 Blood and 24 hour urine samples will be obtained as described in the Pharmacokinetic section above.

8.5 Appropriate x-rays, sonograms, radionuclide scans and contrast-enhanced MRI or CT scans at 8 week intervals for three years, at 12 week intervals for the fourth year, at 16 week intervals for the fifth year, 24 week intervals for the sixth year, then yearly.

8.6 The patient's EKG will be repeated as clinically indicated.

8.7 If the patient is removed from study any clinical or laboratory abnormality that seems possibly related to administration of Antineoplaston will be followed until it resolves or drug-relatedness appears to be unlikely.

9.0 CRITERIA FOR TOXICITY

9.1 Based on previous clinical studies, Antineoplaston therapy can produce the following toxic effects: dizziness, nausea and vomiting; however, the following are considered to be possible additional side effects: anemia, leukopenia, thrombocytopenia, hypocalcemia, hypertension, metabolic acidosis, fluid overload (edema), hypoglycemia, hypokalemia, weakness, fever, myalgias, and abdominal pain. Drug administration requires the use of an indwelling central venous catheter; insertion of this device can be accompanied by local discomfort, pneumothorax, hemothorax, and subclavian artery puncture. Prolonged drug administration by way of the central venous line, can produce infection of that line and phlebitis of the infused blood vessel.

9.2 The NCI Common Toxicity criteria will be used; they are presented in Appendix 1 to this protocol.

9.3 To grade a toxic effect that is covered by the NCI Common Toxicity Criteria, those Criteria will be employed. If the observed toxic effect is not graded within the NCI Common Toxicity Criteria, the following paragraphs provide a general descriptive concept of the toxicity grading system.

Grade 0 - No toxicity

Grade 1 - Mild toxicity, usually transient, requiring no special treatment and generally not interfering with usual daily activities.

Grade 2 - Moderate toxicity which impairs usual activities but may be ameliorated by simple therapeutic maneuvers.

Grade 3 - Severe toxicity which interrupts usual activities and requires therapeutic intervention. Hospitalization may or may not be required.

Grade 4 - Life-threatening toxicity which requires hospitalization.

Any toxicity which causes a drug-related death will be called **Grade 5**.

10.0 CRITERIA FOR THERAPEUTIC RESPONSE

10.1 Protocol response criteria will be based only on objective tumor measurements, and will not include assessment of neurologic status. All tumor measurements must be recorded in centimeters and must have the longest diameter and its perpendicular applied at the widest portion of the tumor recorded. An assessment of response will be made every sixty days. Neurologic status will be assessed independently.

10.2 **Complete Response** - Complete disappearance of all contrast enhancing tumor on neuroimaging studies, and ancillary radiographic studies if appropriate for a minimum

duration of 4 weeks.

10.3 **Partial Response** - More than 50% reduction in the sum of the products of the greatest perpendicular diameters of all measurable lesions, compared to the corresponding baseline evaluation, for 4 weeks or longer. No simultaneous increase in size of any lesion or the appearance of new lesions may occur.

10.4 **Stable Disease** - Less than 50% change (either greater or smaller) in the sum of the products of the perpendicular diameters of the tumor compared to the baseline evaluation. This state must be maintained for a minimum of 12 weeks to qualify for stable disease.

10.6 **Progressive Disease** - Greater than 50% increase in the sum of the products of the greatest perpendicular diameters of the tumor compared to the baseline evaluation. Appearance of significant new lesions will also constitute progressive disease.

10.7 If there is a discrepancy, excluding progression, in the response between the primary tumor site and any metastatic tumor site, the response for each site will be recorded separately.

11.0 CRITERIA FOR REMOVAL FROM STUDY

11.1 Reasons for a patient's removal from the study may include: progression of cancer as described in Section 10.6, severe adverse reactions, intervening medical problems, failure to comply with the protocol, new knowledge which would make continuation of the study unacceptable, the patient's request, and the physician's judgement.

12.0 BIOSTATISTICAL CONSIDERATIONS

12.1 All patients meeting the eligibility criteria will be evaluated for efficacy and toxicity. Analyses of anaplastic astrocytoma and glioblastoma multiforme will be independent, since patients are stratified for these factors at entry to the protocol. Within each stratum a modification of the two-stage Phase II clinical trial design proposed by Fleming will be used in this study.[8] Initially, 15 adequately treated patients will be evaluated in each stratum. If < 1 major response (CR or PR) is observed per stratum, it will be concluded that the true response rate within that stratum is $< 20\%$ with 96% confidence (1-tailed) and no further patients will be entered onto that stratum. If, however, one or more major responses are observed in either stratum, 20 more patients should be accrued to reach a final sample size of 35 adequately treated patients in that stratum. If a total of ≥ 4 responses are observed among the 35 patients/stratum, then there would be sufficient evidence to conclude that the Antineoplaston regimen used is active and merits further study within that diagnostic stratum. This design will detect a response rate = to 20% with a 91% probability and 7% probability of early termination; it will reject a response rate = to 5% with 92% probability and with a 74% probability of early termination.

12.2 Estimated accrual to this protocol based upon adequate drug supplies and past Phase II trials in malignant astrocytomas conducted at Memorial Hospital is two patients monthly.

13.0 REQUIREMENTS FOR REPORTING

13.1 Adverse Drug Reactions

Report by phone to Investigational Drug Branch (IDB) within 24 hours (301-230-2330, available 24 hours, recorder after working hours).

All previously unknown life-threatening or fatal events (Grades 4 and 5) which may be due to drug administration; a written report is to follow within 10 working days.

Written report within 10 working days.

All previously unknown treatment related Grade 2-3 reactions.
All previously known disease related reactions of Grades 4 or 5; lesser drug-related toxicities are submitted as part of study results.

Report in writing within 10 working days to:

Investigational Drug Branch
P.O. Box 30012
Bethesda, MD 20824

13.2 Data

Data will be submitted to CTMS at least every two weeks.

The NCI/DCT Case report program on ACES will be used to report to CTMS.

13.3 Publication of study results

Before the investigators of this study submit a paper or abstract for publication or otherwise publicly disclose informatin concerning Antineoplastons AS2-1 and A10, Dr. Burzynski shall be provided thirty (30) days to review the proposed publication or disclosure to assure that confidential and proprietary data are protected. The investigators should send any proposed publication or disclosure to the Protocl and Information Office of CTEP at the following address:

Protocol and Information Office
Cancer Therapy Evaluation Program
DCT/NCI
Executive Plaza North, Room 730
Bethesda, Maryland 20892

Please note that the NCI Division of Cancer Treatment (DCT) Investigators maintain the full right to the timely publication and presentation of the data from DCT-sponsored studies conducted with Antineoplastons AS2-1 and A10.

14.0 CONSENT PROCEDURES

14.1 All patients will be required to sign a statement of informed consent indicating the investigational nature of this study.

15.0 REFERENCES

1. Antineoplastons: Request for Phase II Trials in CNS Malignancies. CTEP Letter: Rapid Communication Request for Letters of Intent. Vol. 10:10, NCI, DCT, CTEP. June 19,1992.

2. Burzynski SR, Kubove E, Burzysnski B. Phase II clinical trials of antineoplaston A10 and AS2-1 infusions in astrocytoma. *In* Symposium 42 - Novel Differentiation Inducers. Recent Advances in Chemotherapy, Anticancer Section, Proc 17th International Congress of Chemotherapy, Berlin, 1991. Futuramed Publishers, Munich, 1992. pp 2506-2507.

3. Brusilow SW, Danney M, Waber LJ, et al. Treatment of episodic hyperammonemia in children with inborn errors of urea synthesis. N Engl J Med 310:1630-1634, 1984.

4. Brusilow SW. Phenylacetylglutamine may replace urea as a vehicle for waste nitrogen excretion. Ped Res 29:147-150, 1991.

5. Simell O, Sipila I, Rajantie J, Valle DL, Brusilow SW. Waste nitrogen excretion via amino acid acylation: Benzoate and phenylacetate in lysinuric protein intolerance. Ped Res 20:1117-1121, 1986.

6. Samid D, Shack S, Sherman LT. Phenylacetate: A novel nontoxic inducer of tumor cell differentiation. Cancer Res 52:1988-1992, 1992.

7. Brusilow SW, Horwich AL. Urea cycle enzymes. *in* The Metabolic Basis of Inherited Disease, 6th Edition, Scriver CR, Beaudet AL, Sly WS, Valle D. (Editors), McGraw-Hill, New York, 1989, pp 629-644.

8. Fleming TR. One-sample multiple testing procedure for Phase II clinical trials. Biometrics 35:775-784, 1979.

Memorial Sloan-Kettering Cancer Center
1275 York Avenue
New York, New York 10021

STATEMENT OF INFORMED CONSENT FOR CLINICAL RESEARCH

You are being asked to participate in a clinical research study. The doctors at Memorial Hospital study the nature of disease and attempt to develop improved methods of diagnosis and treatment. This is called clinical research. In order to decide whether or not you should agree to be part of this research study, you should understand enough about its risks and benefits to make an informed judgement. This process is known as informed consent.

This consent form gives detailed information about the research study which the doctor will discuss with you. Once you understand the study, you will be asked to sign this form if you wish to participate. You will have a copy to keep as a record.

The research study being proposed to you is:

PHASE II STUDY OF ANTINEOPLASTONS A10 AND AS2-1

IN PATIENTS WITH ADVANCED RECURRENT MALIGNANT ASTROCYTOMAS

PURPOSES OF THE RESEARCH STUDY

The purposes of this research study are: 1) to determine if the administration of Antineoplastons A10 and AS2-1 in your vein will shrink your brain tumor and lead to an overall improvement in your condition; 2) to identify and describe any side effects Antineoplaston treatment; 3) to find out how these Antineoplastons are handled by the body.

You have a malignant glioma, the most common cancerous brain tumor. Despite surgery, radiation and chemotherapy your tumor has come back; without further treatment your tumor will continue to grow, causing neurologic symptoms and disability. At this time treatment with additional surgery or radiation or conventional chemotherapy has only a small chance of slowing the growth of your tumor; accordingly, it is reasonable to consider therapies that are experimental.

Antineoplastons A10 and AS2-1 are chemicals that have been administered to patients with cancer in a private clinic in Texas. The National Cancer Institute (NCI) reviewed the records of 7 patients with brain tumors who were treated at this clinic, and concluded that the treatment justified further study. As a result, NCI is sponsoring clinical trials of Antineoplastons A10 and AS2-1 in patients. The Memorial Sloan-Kettering Cancer Center is participating in those clinical trials. It is hoped that Antineoplastons will prove to be useful anticancer agents, but the success of this treatment cannot be guaranteed.

The two Antineoplastons are being given together in this trial because that is the way that they were used in the treatments reviewed by the National Cancer Institute panel. Although detailed chemical studies have not been performed in patients treated with Antineoplastons, it is known that these Antineoplastons are chemically converted in solution to two other chemicals named phenylacetic acid and phenylacetylglutamine. Phenylacetic acid does slow the growth rate of some cancer cell types in test tubes, including human brain tumor cells. Furthermore, phenylacetic acid has been used for many years in the treatment of children with

conditions other than cancer and is well tolerated.

The dose of Antineoplastons will be based on those used previously. Although these drugs were well tolerated by the patients examined, side effects are possible in the present studies; therefore, all patients will be watched closely for possible reactions. In some patients we will also want to study the way patients remove Antineoplastons from the blood and excrete the component chemicals in the urine. We hope that this information will help us use Antineoplastons safely and effectively.

DESCRIPTION OF THE RESEARCH PROCEDURE

Before presenting this Consent Document to you for your review, we have determined that you are eligible to participate in this study based upon physical examination and tests, which may have included x-rays, CT scans (a special type of x-ray using a computer), MRI scans, nuclear medicine tests, blood and urine laboratory studies. If you are a woman who is pregnant or breast-feeding you must not participate in the treatment. If you are a woman who is capable of becoming pregnant you should have been practicing an acceptable method of birth control for 4 weeks prior to starting the study. You should continue to do so during the study and for at least 4 weeks following completion of the study. Acceptable methods would include a birth control pill, use of a diaphragm, or intrauterine device, or the use of a condom by your sexual partner. If you are a sexually active man you must also be using a condom or other antipregnancy technique to prevent the possibility of a pregnancy developing from sperm that have been damaged by drug treatment.

Antineoplastons will be administered through your vein on a continuing basis over a period of weeks using a programmable portable pump that will permit you to receive much of the therapy at home. In order to minimize the problems of irritation to your veins the infusion will be administered through a a plastic catheter placed in a large vein in the upper part of your chest. This is a frequently used technique in administering medication over a prolonged period. If you do not already have a central venous catheter, it will be necessary to place one in order for you to participate in this study. It is planned that the infusion therapy will be initiated in the hospital where your tolerance to the Antineoplaston therapy will be established. You and your family members will be trained in monitoring the infusion pump and in replacing the plastic bag which contains the Antineoplastons as the infusion of the contents of the previous bag has been completed. Both the pump and the supply of Antineoplastons will be provided to you without charge. In addition to receiving support and advice from this Center, you will be visited in your home by nurses who are trained in infusional therapy.

Although the precise duration of therapy cannot be predicted, it is planned that it will be continued until its effects on your tumor can be determined. It is expected that the majority of patients will receive the infusion for at least a two month period. You may continue to receive Antineoplastons as long as the study lasts, provided that there is no evidence of progression of your cancer, and you are willing to continue to receive therapy. If we detect evidence that the drug is not working against your tumor, it will be stopped and you will be offered suitable alternative therapy. If Antineoplastons produce improvement or stabilization of your cancer (either shrinkage or no further growth), it is planned to continue treatment until such time as you no longer benefit from them.

Pharmacology Studies - Because of the importance of understanding how the body handles Antineoplastons, we will wish to perform pharmacologic studies by obtaining blood and urine from you during the initial days of the infusion. Blood will also be obtained during your visits to the hospital outpatient clinic. The total blood drawn for the purpose of the brief pharmacology study will be 5 ounces.

POSSIBLE SIDE EFFECTS AND RISKS OF THIS TREATMENT PROGRAM

All conventional or experimental treatments for your tumor have potential side effects, including those that may be life threatening. There are risks associated with this study even though the side effects of Antineoplaston administration have been minimal. Reported side effects of the drug's use have included lightheadedness, nausea and vomiting in a small percentage of individuals. The Antineoplastons and their waste products that appear in the urine have a strong and unpleasant odor; this odor may be strong enough to be detectable by family members and social contacts during the treatment period.

Drug administration requires the use of catheter (a plastic tube) placed in a large vein in the upper part of the body; these catheters are widely used to permit administration of a variety of medications to patients. Insertion of the catheter, which requires a brief hospitalization, can be accompanied by local discomfort, and by injury to the blood vessels or the lung resulting in the leakage of blood or air. Prolonged drug administration by way of this central venous catheter, can produce a blood clot in the infused blood vessel that would require therapy. Additionally, central catheters may become infected by bacterial or fungal organisms. Under most circumstances these complications can be treated by administration of anticlotting or antibacterial medications through the catheter. In some patients the device must be removed.

You will be closely observed and efforts made to minimize side effects. If during the course of treatment we become aware of significant drug-induced side effects, we will discuss its effect on your continued participation in the study. If you encounter severe side effects, drug treatments will be interrupted, and consideration given to discontinuing them. You should also be aware that Antineoplaston therapy may involve risks of which we are not currently aware. If you are a woman who may become pregnant during this study, there may be risks to you and to your fetus (unborn child) that are not now known. If you are a sexually active man you should use a condom or other technique to prevent pregnancy in a sexual partner with sperm that may have been affected by this drug treatment.

Some blood samples also will be needed; the side effects are those of punctures into a vein (blood vessel in your arm). The amount of blood being withdrawn for these studies is not considered large enough to require a blood transfusion.

If you are injured as a result of your participation in this research study, emergency care will be made available by the hospital and billed to you as part of your medical expenses. No money will be provided by the hospital as compensation for a research-related injury.

RIGHT TO REFUSE OR WITHDRAW

Your participation in this study is entirely voluntary; the choice to enter, or not to enter this study is yours. You are in a position to make a decision if you understand what the doctor has explained and what you have read about the research study and other possible forms of care. If you decide not to participate, other choices are available to you without prejudice. If you begin the study, you still have a right to withdraw at any time. You will be notified of significant new findings developed during this study that may influence your willingness to continue participation on this study. If you should withdraw, you will be offered other available care which suits your needs and medical condition. Such alternative therapy could include other forms of chemotherapy with other experimental or conventional drugs, radiation therapy, or close observation without specific anticancer treatment.

BENEFIT OF PARTICIPATION

Although we hope that this research study will be of benefit to you, and that the information derived from your participation in it will help others, we cannot say that it will be directly beneficial to you.

FINANCIAL COST OF PARTICIPATION

There will be no charge to you for the Antineoplastons or for the infusion pump. However, you will be responsible for the costs of hospitalization and the home infusion visits. There will be a conventional charge for laboratory tests necessary to assure safety and to check for therapeutic effects of treatment. There will be no charge for the pharmacology tests of blood or urine.

PRIVACY

Your research and hospital records that identify you by name will be maintained in strict confidence, except that they may be inspected by the sponsor of the study (the National Cancer Institute), the Food and Drug Administration, and other government agencies; they will not otherwise be released except by law.

INSTITUTIONAL REVIEW BOARD

This Board is legally responsible for making sure that research with patients is appropriate and that the patient's rights and welfare are protected; that Board has reviewed this protocol.

QUESTIONS

The physician in charge of this research study is: Dr. Mark Malkin, Telephone number (212) 639-6688. If you need more information about this study before you decide to join, or at any other time, you may contact these physicians. In the event that you do decide to participate, they should also be called if there are side effects from the research study. A non-physician whom you may call for information about the consent process, research patient's rights, or research-related injury is Pat Keating, telephone number (212) 639-7202.

PATIENT INFORMED CONSENT FOR CLINICAL RESEARCH

Title: Phase II Study of Antineoplastons A10 and AS2-1 in Patients with Advanced Recurrent Malignant Astrocytomas.

Purpose: The purposes of this research study are to find out whether the administration of Antineoplastons in the vein will reduce the size of your brain tumor and help you, to describe any side effects of this treatment, and to find out how Antineoplastons are handled by the body.

STATEMENT OF PHYSICIAN OBTAINING INFORMED CONSENT

I have fully explained this research study to this patient. In my judgement and the patient's, there was sufficient access to information, including risks and benefits, to make an informed decision.

Date:_____ Physician's Signature:_____

Physician's Name:_____
(Print)

PATIENT'S STATEMENT

I have read the description of the clinical research study or have had it translated into language I understand. I have also talked it over with the doctor to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form to keep for myself.

Patient Number:_____ Patient Name:_____
(Print)

Patient's Signature:_____

Date:_____

COMMON TOXICITY CRITERIA

		GRADE			
TOXICITY	0	1	2	3	4
Blood/Bone Marrow					
WBC	≥4.0	3.0 - 3.9	2.0 - 2.9	1.0 - 1.9	<1.0
PLT	WNL	75.0 - normal	50.0 - 74.9	25.0 - 49.9	<25.0
Hgb	WNL	10.0 - normal	8.0 - 10.0	6.5 - 7.9	<6.5
Granulocytes/ Bands	≥2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	<0.5
Lymphocytes	≥2.0	1.5 - 1.9	1.0 - 1.4	0.5 - 0.9	<0.5
Hemorrhage (clinical)	none	mild, no transfusion	gross, 1-2 units transfusion per episode	gross, 3-4 units transfusion per episode	massive, >4 units transfusion per episode
Infection	none	mild	moderate	severe	life-threatening
Gastrointestinal					
Nausea	none	able to eat reasonable intake	intake significantly decreased but can eat	no significant intake	--
Vomiting	none	1 episode in 24 hrs	2-5 episodes in 24 hrs	6-10 episodes in 24 hrs	>10 episodes in 24 hrs, or requiring parenteral support
Diarrhea	none	increase of 2-3 stools/day over pre-Rx	increase of 4-6 stools/day, or nocturnal stools, or moderate cramping	increase of 7-9 stools/day, or incontinence, or severe cramping	increase of ≥10 stools/day or grossly bloody diarrhea, or need for parenteral support
Stomatitis	none	painless ulcers, erythema, or mild soreness	painful erythema, edema, or ulcers, but can eat	painful erythema, edema, or ulcers, and cannot eat	requires parenteral or enteral support
Liver					
Bilirubin	WNL	--	<1.5 x N	1.5 - 3.0 x N	>3.0 x N
Transaminase (SGOT, SGPT)	WNL	≤2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	>20.0 x N
Alk Phos or 5'nucleotidase	WNL	≤2.5 x N	2.6 - 5.0 x N	5.1 - 20.0 x N	>20.0 x N
Liver-- clinical	no change from baseline	--	--	precoma	hepatic coma

COMMON TOXICITY CRITERIA (continued)

	TOXICITY	GRADE 0	1	2	3	4
Kidney, Bladder	Creatinine	WNL	< 1.5 x N	1.5 - 3.0 x N	3.1 - 6.0 x N	>6.0 x N
	Proteinuria	no change	1+ or <0.3 g% or <3 g/l	2 - 3+ or 0.3 - 1.0 g% or 3 - 10 g/l	4+ or >1.0 g% or >10 g/l	nephrotic syndrome
	Hematuria	neg	micro only	gross, no clots	gross + clots	requires transfusion
	Alopecia	no loss	mild hair loss	pronounced or total hair loss	--	--
	Pulmonary	none or no change	asymptomatic, with abnormality in PFT's	dyspnea on significant exertion	dyspnea at normal level of activity	dyspnea at rest
Heart	Cardiac dysrhythmias	none	asymptomatic, transient, requiring no therapy	recurrent or persistent, no therapy required	requires treatment	requires monitoring; or hypotension, or ventricular tachycardia, or fibrillation
	Cardiac function	none	asymptomatic, decline of resting ejection fraction by less than 20% of baseline value	asymptomatic, decline of resting ejection fraction by more than 20% of baseline value	mild CHF, responsive to therapy	severe or refractory CHF
	Cardiac-- ischemia	none	non-specific T-wave flattening	asymptomatic, ST and T wave changes suggesting ischemia	angina without evidence for infarction	acute myocardial infarction
	Cardiac-- pericardial	none	asymptomatic effusion, no intervention required	pericarditis (rub, chest pain, ECG changes)	symptomatic effusion; drainage required	tamponade; drainage urgently required
Blood Pressure	Hypertension	none or no change	asymptomatic, transient increase by greater than 20 mm Hg (D) or to >150/100 if previously WNL. No treatment required	recurrent or persistent increase by greater than 20 mm Hg (D) or to >150/100 if previously WNL. No treatment required	requires therapy	hypertensive crisis
	Hypotension	none or no change	changes requiring no therapy (including transient orthostatic hypotension)	requires fluid replacement or other therapy but not hospitalization	requires therapy and hospitalization; resolves within 48 hrs of stopping the agent	requires therapy and hospitalization for >48 hrs after stopping the agent

COMMON TOXICITY CRITERIA (continued)

		GRADE			
TOXICITY	0	1	2	3	4
Local	none	pain	pain and swelling, with inflammation or phlebitis	ulceration	plastic surgery indicated
Weight gain/loss	<5.0%	5.0 - 9.9%	10.0 - 19.9%	≥20.0%	--
Metabolic: Hyperglycemia	<116	116 - 160	161 - 250	251 - 500	>500 or keto-acidosis
Metabolic: Hypoglycemia	>64	55 - 64	40 - 54	30 - 39	<30
Metabolic: Amylase	WNL	<1.5 x N	1.5 - 2.0 x N	2.1 - 5.0 x N	>5.1 x N
Metabolic: Hypercalcemia	<10.6	10.6 - 11.5	11.6 - 12.5	12.6 - 13.5	≥13.5
Metabolic: Hypocalcemia	>8.4	8.4 - 7.8	7.7 - 7.0	6.9 - 6.1	≤6.0
Metabolic: Hypomagnesemia	>1.4	1.4 - 1.2	1.1 - 0.9	0.8 - 0.6	≤0.5
Coagulation: Fibrinogen	WNL	0.99 - 0.75 x N	0.74 - 0.50 x N	0.49 - 0.25 x N	≤0.24 x N
Coagulation: Prothrombin time	WNL	1.01 - 1.25 x N	1.26 - 1.50 x N	1.51 - 2.00 x N	>2.00 x N
Coagulation: Partial thromboplastin time	WNL	1.01 - 1.66 x N	1.67 - 2.33 x N	2.34 - 3.00 x N	>3.00 x N

COMMON TOXICITY CRITERIA (continued)

TOXICITY	GRADE 0	1	2	3	4
Neuro-- sensory	none or no change	mild paresthesias, loss of deep tendon reflexes	mild or moderate objective sensory loss; moderate paresthesias	severe objective sensory loss or paresthesias that interfere with function	--
Neuro-- motor	none or no change	subjective weakness; no objective findings	mild objective weakness without significant impairment of function	objective weakness with impairment of function	paralysis
Neuro-- cortical	none	mild somnolence or agitation	moderate somnolence or agitation	severe somnolence, agitation, confusion, disorientation, or hallucinations	coma, seizures, toxic psychosis
Neuro-- cerebellar	none	slight incoordination, dysdiadokinesis	intention tremor, dysmetria, slurred speech, nystagmus	locomotor ataxia	cerebellar necrosis
Neuro-- mood	no change	mild anxiety or depression	moderate anxiety or depression	severe anxiety or depression	suicidal ideation
Neuro-- headache	none	mild	moderate or severe but transient	unrelenting and severe	--
Neuro-- constipation	none or no change	mild	moderate	severe	ileus >96 hrs
Neuro-- hearing	none or no change	asymptomatic, hearing loss on audiometry only	tinnitus	hearing loss interfering with function but correctable with hearing aid	deafness not correctable
Neuro-- vision	none or no change	--	--	symptomatic subtotal loss of vision	blindness
Skin	none or no change	scattered macular or papular eruption or erythema that is asymptomatic	scattered macular or papular eruption or erythema with pruritus or other associated symptoms	generalized symptomatic macular, papular, or vesicular eruption	exfoliative dermatitis or ulcerating dermatitis
Allergy	none	transient rash, drug fever <38c, 100.4F	urticaria, drug fever =38c, 100.4F mild bronchospasm	serum sickness, bronchospasm, req parenteral meds	anaphylaxis
Fever in absence of infection	none	37.1 - 38.0c 98.7 - 100.4F	38.1 - 40.0c 100.5 - 104.0F	>40.0c >104.0F for less than 24 hours	>40.0c (104.0F) for more than 24 hrs or fever accompanied by hypotension

urologic

DCT GUIDELINES FOR MULTICENTER INVESTIGATIONAL AGENT STUDIES

Section

DCT GUIDELINES FOR MULTICENTER INVESTIGATIONAL AGENT STUDIES

1.0 PROTOCOL DEVELOPMENT

1.1 For each multicenter study, one institution shall be designated as the "Coordinating Center."

1.2 The Protocol Chairman at the Coordinating Center will be the single liaison with the CTEP Protocol and Information Office (PIO). The Protocol Chairman will coordinate the development, submission, and approval of the protocol as well as its subsequent amendments, results reports, and publications.

2.0 PROTOCOL DOCUMENT

There will be only one version of the protocol and each participating institution will use that document. It should not be rewritten or modified by any one other than the Protocol Chairman at the Coordinating Center who is solely responsible for obtaining CTEP approval and distributing the protocol to all participants, as well as formulating protocol amendments for CTEP approval and distributing protocol amendments to all participants.

2.1 Protocol Title Page

The protocol document developed for a multicenter study should contain the following information on the title page:

1) Date of Document
2) Title of Study
3) Protocol Chairman, including name, institution, address and phone number.
4) Name of each participating institution, and Responsible Investigator at each (with phone number)
5) NCI number, local protocol numbers
6) List of DCT-supplied investigational agent(s) and NSC number(s)

The multicenter protocol facesheet should be updated whenever new institutions are added.

2.2 Patient Entry Procedure: Patients should be centrally registered by telephone with the Coordinating Center. The protocol document should specify directions, including registrar's name and phone number. Registration procedures should include a check of eligibility and regulatory issues (see quality Assurance).

2.3 Records to be Kept: The Coordinating Center is responsible for developing common case report forms, and all data should be submitted to the Coordinating Center on these forms. The forms to be used should be submitted with the protocol. The protocol document should specify the for, submission schedule and where to send the data.

2.4 ADR Reporting: There are two options for the flow of Adverse Drug Reaction (ADR) Reporting:

1) Investigator reports simultaneously to Protocol Chairman and the Investigational Drug Branch (IDB) DCT.

2) Investigator reports to Protocol Chairman who reports to IDB.

The selected procedure for ADR reporting must be specified in the protocol document, along with types of reactions to be reported and timing of reports.

1) Report by phone or FAX to Investigational Drug Branch (IDB) within 24 hours (301 230-2330 or FAX 301 230-0159).

   a. All life-threatening and lethal (Grade 4 and 5) unknown reactions. Written report to follow within ten working days.

2) Report in writing within 10 working days.

   a. Life-threatening and lethal (Grade 4 and 5) known reactions (except Grade 4 myelosuppression.

   b. Grade 2 and 3 unknown reactions.

3) Address for submitting ADR reports.

Investigational Drug Branch
P.O. Box 30012
Bethesda, Maryland 20024

All written reports should be submitted on the NCI ADR Report Form.

3.0 PROTOCOL SUBMISSION TO CTEP:

3.1 The Protocol and Information Office, (PIO) will assign an NCI protocol number when a protocol first arrives in CTEP. Once the protocol is approved, the number is "a constant" which ties together recordkeeping in CTEP: the PIO files, the drug ordering and distribution system, the filing of the protocol and amendments in INDs to the FDA, ADR reporting as well as submissions to PDQ. Therefore, this NCI protocol number should appear on the protocol facesheet and in all subsequent correspondence and reports. On the protocol and each amendment, on the drug orders, status notices, ADR reports and publications.

3.2 When submitting a new protocol for review, please identify the study as a multicenter study. The cover letter should explain any background information that may be useful to the protocol Review Committee, but not appropriate in the body of the document.

3.3 When the protocol is initiated as a single institution study and it is necessary to change to a multicenter, the Coordinating Center should submit a protocol amendment requesting the additional participants, as well as a revised document addressing each logistical requirement for multicenter trials.

4.0 STUDY CONDUCT

4.1 The protocol will be conducted as a single research effort and data from each participant will be included in the analysis of results.

4.2 The Protocol Chairman will be responsible for the conduct of the study and the monitoring of the progress; he/she will review all case report forms from each participant; uncritical acceptance of summary data alone from other institutions is not sufficient.

5.0 QUALITY ASSURANCE:

5.1 The individual accepting registrations should ascertain the date of IRB approval at each participating institution before registering the first patient from that institution.

5.2 During the registration call, eligibility criteria should be reviewed. The registrar should ascertain that an informed consent document has been signed before registering each patient.

5.3 Multicenter study records at each participating institution will be randomly selected for audit when that institution is scheduled for periodic on-site audits by DCT and its Clinical Trials Monitoring Service (at least once every three years).

6.0 DRUG ORDERING:

The NCI assigned protocol number should be used for ordering investigational agents. Orders should be submitted directly from each participating institution

7.0 PROTOCOL AMENDMENTS/STATUS CHANGES:

7.1 Each change to the protocol must be organized and documented by the Coordinating Center. The Protocol Chairman at the Coordinating Center should submit the amendment to CTEP for approval and then distribute it to study participants.

7.2 The amendment should be written so that no other institution needs to reformat the information but can simply copy and distribute. Amendments need to display the NCI protocol number. The changes must be clearly outlined and supported by amended protocol pages or replacement protocol document.

7.3 The coordinating Center should keep CTEP abreast of each study status change by submitting the information on the Protocol Submission Checklist to the PIO.

8.0 RESULTS REPORTING:

The Protocol Chairman at the Coordinating Center will be responsible for submitting study results to CTEP as required.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Stanislaw R. Burzynski

Serial No.: 09/121,567

Filed: July 23, 1998

For: TREATMENT REGIMEN FOR ADMINISTRATION OF PHENYLACETYLGLUTAMINE, PHENYLACETYLISOGLUTAMINE, AND/OR PHENYLACETATE

Group Art Unit: 1614

Examiner: J. Goldberg

Atty. Dkt. No: 10379.0047.NPUS00
(BURG047/KAM)

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SUPPLEMENTAL DECLARATION OF STANISLAW R. BURZYNSKI, M.D., PH.D. UNDER 37 C.F.R. § 1.132

Supplemental to the Declaration filed August 28, 2000, I, Stanislaw R. Burzynski, do hereby declare that:

1. As one of the co-authors, I am completely familiar with the reference entitled "Preclinical studies on antineoplaston A10 injections" by A.Q. Ashraf, M.C. Liau, M.O. Mohabbat, and S.R. Burzynski, *Drugs Exptl. Clin. Res. Suppl. 1,* 1986, 12:37-45 ("Ashraf *et al.*). I am also familiar with the reference entitled "Inhibitory Effect of antineoplaston A10 and AS2-1 on human hepatocellular carcinoma" by H. Tsuda, A. Iemura, M. Sata, M. Uchida, K. Yamana, and H. Hara, *The Kurume Medical* Journal, 1986, 43:137-147, ("Tsuda *et al.*"). I have thoroughly reviewed and understand the contents of both the Ashraf *et al.* and the Tsuda *et al.* references.

2. I am familiar with the distinctions between the invention disclosed and claimed in U.S. Patent Application Serial Number 09/121,567 (the '567 application) and the

materials disclosed by the Ashraf *et al.* and Tsuda *et al.* references, taken both individually and/or in combination.

3. Based on a thorough reading and understanding of the '567 application, the Ashraf *et al.* reference, and the Tsuda *et al.* reference, I can state that the compositions and methods disclosed and claimed in the '567 application provide surprising and unexpectedly superior efficacy (as defined by the FDA approved efficacy standards described in paragraph 11 of the Declaration I filed August 28, 2000) for the treatment of neoplastic disease when compared with the materials and methods disclosed by a combination of the Ashraf *et al.* and Tsuda *et al.* references.

4. The instantly claimed invention provides for the use of concentrated A10. A10 comprises a 4:1 ratio, by mass, of phenylacetylglutamine (PAG) to phenylacetylisoglutamine (PAIG). For concentrated A10, the combined concentration of the PAG and PAIG is about 200-350 mg/mL. In contrast the Ashraf *et al.* reference describes only dilute A10, wherein the concentration of PAG and PAIG is only 100 mg/mL.

5. Typically in the treatment of cancer, and other neoplastic disease, chemotherapeutic drugs are delivered in dilute solutions in order to minimize their negative effects to the patient. Contrary to conventional practice, which mandates using low concentrations of chemotherapeutic drugs, I have demonstrated the advantages of using concentrated A10 to treat neoplastic disease. It is my belief that applying such high concentrations of drug, as described and claimed in the instant application, is a completely new and innovative concept.

6. The advantages of using concentrated as opposed to diluted A10 are at least two-fold. First, with dilute A10, as with other standard chemotherapeutic agents, patients often experience increased fluid retention. This fluid retention usually leads to swelling of the extremities, accumulation of fluid in the pleural, peritoneal, and pericardial cavities, and swelling of the target tumor. The fluid retention caused by treatment with dilute A10 frequently required either discontinuation of the treatment or the

administration of diuretics. In contrast, I have found that the administration of concentrated A10 leads to increased elimination of urine. Increased urine elimination allows the patient to decrease edema, pleural effusion, ascites, and swelling around the tumor. This effect is especially important in the treatment of malignant brain tumors, where swelling around the tumor may increase as a result of tumor necrosis and cause severe complications, including death. Significantly, the diuretic effect exhibited by patients treated with concentrated A10 is not observed in patients treated with dilute A10.

7. Second, another advantage of using concentrated A10 is the enhanced elimination of waste chemicals, such as uric acid, which are the byproduct of the dying tumor. Accumulation of such chemicals in the blood may result in "*tumor lysis syndrome,*" a severe complication associated with cancer treatment. This syndrome requires the discontinuance of chemotherapeutic treatment and may cause the patient's death. The increased urine elimination provided by the administration of concentrated A10 helps prevent the onset of tumor lysis syndrome by greatly facilitating the elimination of the toxins from the blood, via excretion through the kidneys. This benefit is not provided by the administration of dilute A10.

8. A final advantage of using concentrated A10 at the high effusion rates, taught in the instant claims, is that it allows the drug to penetrate the tumor in higher concentrations, thereby providing a markedly improved tumor reduction, as demonstrated by the studies reported in Figure 1 of the Declaration filed on August 28, 2000.

9. Figure 1 summarizes data which show only a 5.5% objective response rate for the Burzynski and Kubove study (third column form the left), which was performed using dilute A10 (100 mg/mL), as described in Ashraf *et al.* In comparison, 48.6%, 54.6%, and 62.5% objective response rates were achieved using concentrated A10 (final three columns on the right of the figure). Thus, this analysis provides a direct comparison, between the results observed using dilute A10 described by Ashraf *et al.* and the results achieved with the instantly claimed concentrated A10. This comparison clearly exhibits the superior efficacy of concentrated A10 for the treatment of neoplastic disease as when

compared with the dilute A10 described in Ashraf *et al.* and Tsuda *et al.* (greater than a 10-fold more effective).

10. None of the therapeutic advantages provided by concentrated A10 are provided by dilute A10. Furthermore, there is nothing in the combined teaching of Ashraf *et al.* and Tsuda *et. al.* which would render these advantages obvious. Often, cancer treatment regimens are carried out with an amount of the therapeutic agent which is only slightly below the lethal dosage. These barely sub-lethal doses are frequently necessary in order to deliver enough of the chemotherapeutic to eliminate the cancerous cells. Therefore, for the chemotherapy drugs currently in use, a dramatic increase in dosage, such as 25-40 times, would likely result in the death of the patient.

11. As stated in paragraph 6., above, clinical treatment with dilute A10, the composition described in Ashraf *et al.* and Tsuda *et al.*, often led to fluid retention which required discontinuing treatment or the administration of diuretics, in order to prevent harm to the patient. Since treatment with dilute A10 frequently produced fluid retention and edema, one would not predict that a much higher concentration would achieve beneficial outcomes. However, I have discovered that, contrary to what would be predicted by an artisan of ordinary skill, treatment with concentrated A10, 2.5 to 3.5 times more concentrated than dilute A10, does not significantly increase toxicity to the patient, except for the possibility of dehydration, instead it provides increased drug delivery to the tumor.

12. Consequently, I conclude that the presently claimed invention is both novel and unobvious over all previous art. More specifically, I conclude that an ordinarily skilled artisan would be convinced that the presently claimed invention is both novel and unobvious with respect to the teachings of Ashraf *et al.* and Tsuda *et. al.*, taken either separately or in combination.

13. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the

like so made are punishable by fine or imprisonment or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

JANUARY 18, 2001

Date

Stanislaw R. Burzynski

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ORIGINAL ARTICLE

Inhibitory Effect of Antineoplaston A10 and AS2-1 on Human Hepatocellular Carcinoma

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Summary: Antineoplastons, first described by Burzynski, are naturally occurring peptides and aminoacid derivatives which control neoplastic growth. Antineoplaston A10 (3-phenylacetylamino-2,6-piperidinedione) is the first chemically identified antineoplaston and when it is administered orally it is hydrolysed in pancreatic juice to phenylacetylglutamine and phenylacetylisoglutamine in the ratio of 4 to 1. These metabolites are water soluble and have antitumor effect, they are further degraded to phenylacetic acid. The mixture of phenylacetylglutamine and phenylacetylisoglutamine in the ratio of 4 to 1 was formulated as Antineoplaston A10 injectable formulation. The mixture of phenylacetylglutamine and phenylacetic acid in the ratio of 1 to 4 was also shown to have antitumor effect in tissue culture study, then formulated as Antineoplaston AS2-1. The reported cytostatic inhibitory effect of A10 on human hepatocellular carcinoma cells and differentiation inducing effect of AS2-1 on various tumor cells suggest potential benefit for the treatment of human hepatocellular carcinoma since this tumor recurs frequently despite initial successful treatment. We report here the effects of Antineoplaston A10 and AS2-1 on cell proliferation, cell morphology, cell cycle, and DNA in human hepatocellular carcinoma cell lines. Both agents inhibited cell proliferation and increased the number of cells in G_0 and G_1 phases and Antineoplaston AS2-1 induced apoptosis, we also describe our clinical experience of a hepatocellular carcinoma (HCC) patient whose tumor, after incomplete transcatheter arterial embolization (TAE) for a 7 cm*7 cm HCC, has been stable for more than 15 months during which time he has been taking Antineoplaston AS2-1 continuously without any serious adverse effects.

Key words: Antineoplaston A10, AS2-1, hepatocellular carcinoma, cytostasis, apoptosis

Introduction

Hepatocellular carcinoma (HCC) has become one of the leading causes of death from cancer in Japan. Despite of early detection, the prognosis of HCC is not yet satisfactory. Early small tumors can often be treated successfully by percuta-

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neous ethanol injection therapy (PEIT), transcatheter arterial embolization (TAE), microwave coagulation necrosis (MCN), or surgery but recurrence is frequent, and often multicentric within a year (Nakao et al. 1991).

Anticancer therapy to prevent multiple recurrence in HCC requires the intrahepatic arterial infusion of chemotherapeutic agents. This procedure is often stressful to the liver which shows dysfunction because of underlying chronic hepatitis or cirrhosis. The efforts must be made to develop nontoxic, chemopreventive agents effective against such recurrence. Antineoplaston A10, the first chemically identified and synthesized antineoplaston, exhibits a chemopreventive effect against chemicals and virus induced tumor formation, as well as, an inhibitory effect on tumor growth (Burzynski, 1976; Burzynski and Hai, 1985; Eriguchi et al. 1988; Muldoon et al. 1988; Nishida et al. 1991).

Antineoplaston AS2-1, the degradation products of Antineoplaston A10, induces cell differentiation in the HL-60, erythroleukemia, malignant melanoma, and fibrosarcoma cells (Burzynski et al. 1986; Samid et al. 1991, 1992). Antineoplaston A10 and AS2-1 exert cytostatic action on tumor cells rather than cytotoxic. It thus appears that Antineoplaston A10 and AS2-1 may be of value in preventing recurrence in HCC.

Accordingly, we tested the effect of Antineoplaston A10 and AS2-1 on 6 human hepatocellular cell lines. We also describe our clinical experience of a HCC patient whose tumor has been stable for more than 15 months with Antineoplaston AS2-1 treatment after incomplete TAE was performed for a 7 cm* 7 cm HCC.

Materials and Methods

Cell lines and cell culture

Six human hepatocellular carcinoma cell lines (KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, HAK-1B), established at the First Department of Pathology, Kurume University School of Medicine, were subjected to an in vitro study. The cells were maintained at 37℃ in an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 5% fetal bovine serum (Whitvineker Bioproducts, Walkersville, MD USA), 100 units/ml penicillin, 100 μg /ml streptomycin (GIBCO, Chargin Falls, OH USA) as a basal medium.

Effect of Antineoplaston A10 and AS2-1 on cell proliferation

An appropriate number of cells from each cell lines was seeded on 96-well plastic plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ USA) and cultured with basal medium for one day. On the next day, the medium was exchanged for fresh medium containing various concentrations (0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/ml) of Antineoplaston A10 or AS2-1 (Burzynski Research Institute, Houston, Texas USA). The cells were cultured for 48 or 72 hs, and then MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-2H-bromide) (Chemicon, Temecula, CA USA) was added to the medium in each well to give a final concentration of 250 μg/ml and the plates were incubated for 4 hs at 37℃. The supernatants were gently removed, 100 μl/well of 40 mM HCL/dimethylsulfoxide

was added to dissolve the resulting pigment. Viable cell numbers were estimated by measuring the absorbance with an Easy Reader EAR 400 (SLT Lab Instruments, Saltzburg, Austria), with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Analysis of cell morphology

Cells were seeded on Lab-Tek tissue culture chamber slides (Miles Laboratories, Naperville, IL USA) and cultured with basal medium for one day. On the next day, the medium was exchanged for fresh medium containing Antineoplaston A10 (8 mg/ml) or AS2-1 (2 and 4 mg/ml). The cells were cultured for 72 hs. Then they were fixed in Carnoy's solution and stained with Hematoxylin-Eosin for microscopic study.

Analysis of cell cycle and DNA synthesis

Cells were seeded on a 60 mm plastic dish (Falcon) and cultured with basal medium for one day. On the next day, the medium was exchanged for fresh medium containing Antineoplaston A10 (8 mg/ml) or Antineoplaston AS2-1 (2, 4 mg/ml) and the cells were cultured for 72 hs. Cells were then labeled with 10 μM bromo-2'-deoxyuridine (BrdU) at 37℃ for 30 min, washed with PBS, pH 7.6, detached with Trypsin-EDTA, and fixed with 70% cold ethanol at 4C overnight. After fixation, the cells were treated with 2N HCL/0.5% Triton X 100 for 20 min to denature the double strand DNA and to lyses the cell membrane. After neutralization with 0.1M Na2B4O7/PBS, pH 8.5, the cells were washed with 0.5% Tween 20/PBS, incubated with 20 μl anti-BrdU antibody (Becton Dickinson Immunocytometry Systems USA, San Jose, CA USA) for 30 min at room temperature, washed twice with 0.5% Tween 20/PBS, incubated for 20 min with 20 μl fluorescein conjugated goat anti-mouse immunoglobulin (Becton Dickinson Immunocytometry Systems), and washed with PBS. Then DNA was stained with 5 μg/ml propidium iodide and flow cytometric analyses were performed with a FACScan (Becton Dickinson Immunocytometry System).

Analysis of DNA

KIM-1 and HAK-1A cells were seeded on 10 cm plastic dishes (Falcon) and cultured with basal medium for several days to subconfluence and medium was exchanged for fresh medium containing Antineoplaston A10 (8 mg/ml) or AS2-1 (2 and 4 mg/ml) or for medium without Antineoplaston, and the cells were cultured for 72 hs. The cells were then harvested with a cell scraper and extraction of genomic DNA was performed with Sepa Gene (Sanko Junyaku Co., Ltd., Tokyo, Japan). The DNA was dissolved in Tris/EDTA buffer, and 20 μg per lane of sample DNA or DNA molecular size marker (X174/Hae Ⅲ digest) was electrophoresed in 1.6% agarose gel and visualized with ethidium bromide under UV illumination.

Clinical application of Antineoplaston AS2-1 in HCC patient

A 50-year-old male patient underwent TAE with Lipiodol, ADM for a 7 cm *7 cm HCC located in right hepatic lobe. Three months later, dynamic CT disclosed early enhancement and low attenuation in the delay phase around the embolized area suggesting the growth of a viable

tumor lesion. The patient again underwent TAE but a viable tumor lesion was still shown on dynamic CT. Four months later after this first TAE, he joined the Antineoplaston AS2-1 phase I study program which has been conducted at Kurume University Hospital with the approval of the Ethics Committee since 1988. He signed an informed consent form, after which, treatment with oral formulation of Antineoplaston AS2-1 8 g-10 g/day was instituted. An echogram was taken every month and CT was performed every 3 months. Hematological, urological, and liver function tests were performed every 3 months. He was treated as an outpatient except for a one week admission for PEIT, this being undertaken to treat a small suspicious nodule that appeared on the echogram after 6 months continuous treatment with Antineoplaston AS2-1 8-10 g/day.

Results

Effects on cell proliferation

The optic density of the 6 HCC cell lines cultured for 48 or 72 hs in medium containing Antineoplaston A10 (2, 4, 8 mg/ml) or AS2-1 (0.5, 1, 2 mg/ml) is shown in Fig. 1. Antineoplaston A10 and AS2-1 inhibited cell proliferation time- and dose-dependently in all 6 HCC cell lines. Antineoplaston AS2-1 inhibited cell proliferation markedly regardless of the degree of differentiation of hepatocellular carcinoma cell and no viable cells were found at an concentration of 8 mg/ml (data not shown). A concentration of 8 mg/ml of Antineoplaston A10 was required to inhibit cell proliferation in KIM-1, KYN-3, HAK-1A, and HAK-1B cells.

Effects on cell morphology

Many apoptotic cells characterized by cytoplasmic atrophy, nuclear contraction, and irregular aggregation of nuclear chromatin, were observed in HAK-1A, KIM-1, and HAK-1B cells treated by Antineoplaston AS2-1 at concentrations of 2 and 4 mg/ml, apoptotic cells were not observed in cells treated with Antineoplaston A10 at 8 mg/ml (Fig. 2).

Effects on cell cycle

Antineoplaston A10 and AS2-1 reduced the BrdU uptake ratio and the number of cells in G_2/M phase in KIM-1, KYN-1, KYN-2, and HAK-1A cells. Cells in the D_1 region, not uptaking BrdU between 2C and 4C DNA contents, were observed in KYN-1 and HAK-1A cells with Antineoplaston AS2-1 at a concentration of 4 mg/ml (Fig. 3).

Agarose gel electrophoresis of DNA from KIM-1 and HAK-1A cells showed DNA fragmentation with ladder in cells treated with Antineoplaston AS2-1 at concentrations of both 2 and 4 mg/ml (Fig. 4), no DNA fragmentation was observed in the cells treated with Antineoplaston A10 at 8 mg/ml.

Dynamic CT findings in the patient during the clinical course

The viable part of the HCC lesion, high in early phase and low in delay phase attenuating lesion around embolized area, was recognized on dynamic CT taken 3 months after the first TAE. It disappeared gradually shown in the CT findings 13 and 20 months after the patient had begun treatment with Antineoplaston AS2-1 (Fig. 5).

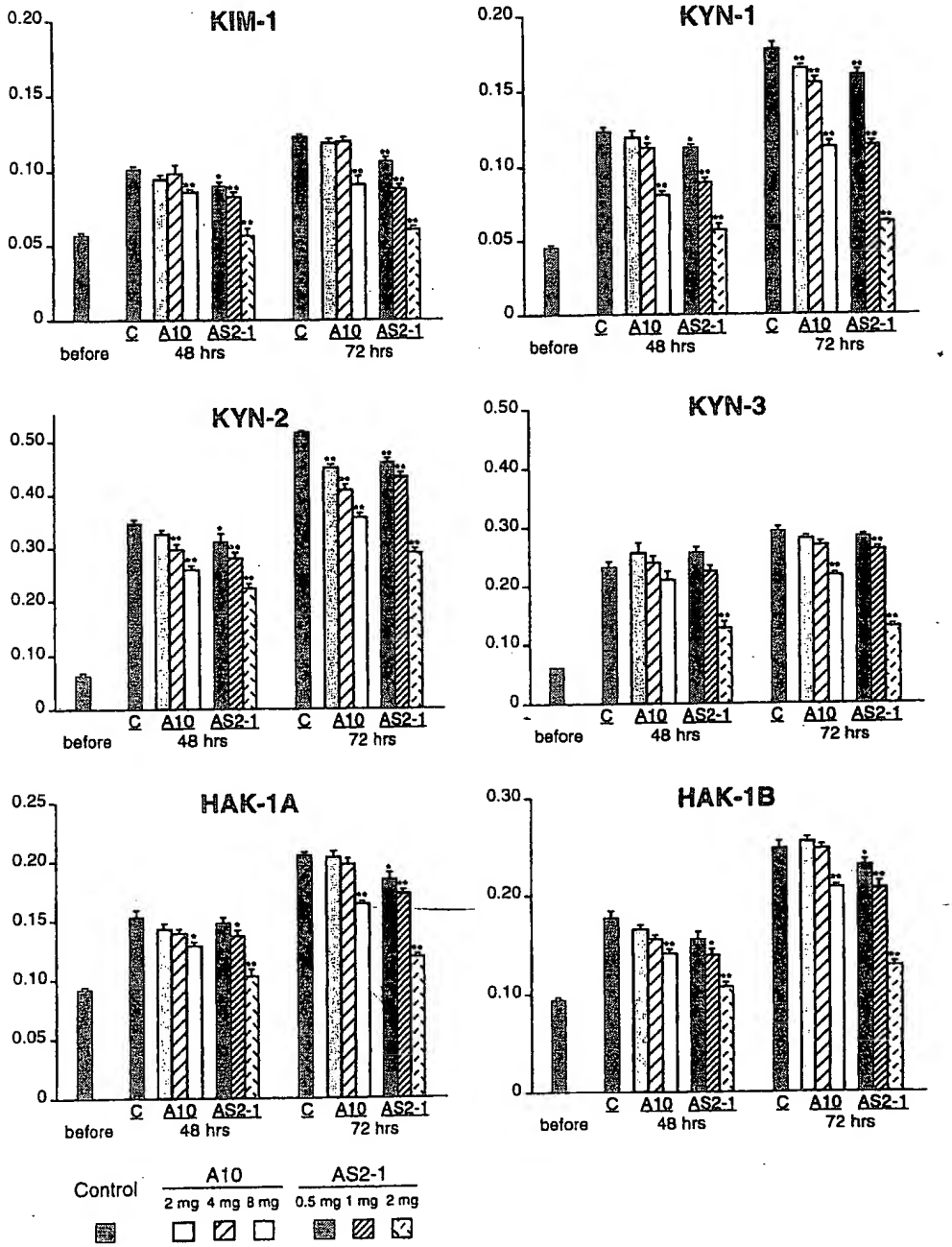

Fig. 1. Optic Density in MTT assay for KIM-1, KYN-1, KYN-2, KYN-3, HAK-1A, HAK-1B hepatocellular carcinoma cells incubated for 48, 72 hs with Antineoplaston A10 at concentrations of 2 mg/ml (□), 4 mg/ml (▨), 8 mg/ml (□) and with Antineoplaston AS2-1 at concentrations of 0.5 mg/ml (▩), 1 mg/ml (▨), 2 mg/ml (▨). (▩) indicates control. *,** represent $p<0.05$, $p<0.01$ vs corresponding for control in each time points (Mann-Whitney U test). Antineoplaston A10 and AS2-1 inhibited cell growth dose-dependently.

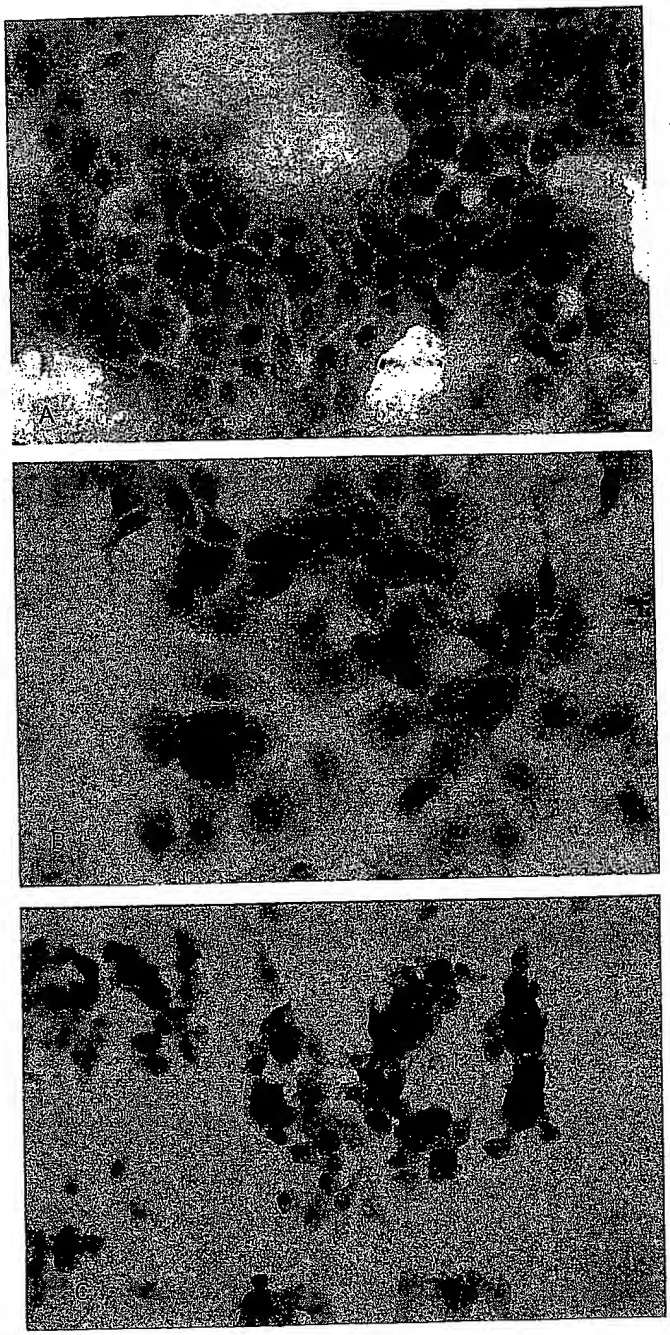

Fig. 2. H. E. stained KIM-1 hepatocellular carcinoma cells treated with Antineoplaston AS2-1 at concentrations of 2 mg/ml (B), 4 mg/ml (C) for 72 hs. A indicates control. Apoptotic cells were observed in AS2-1 treated cells.

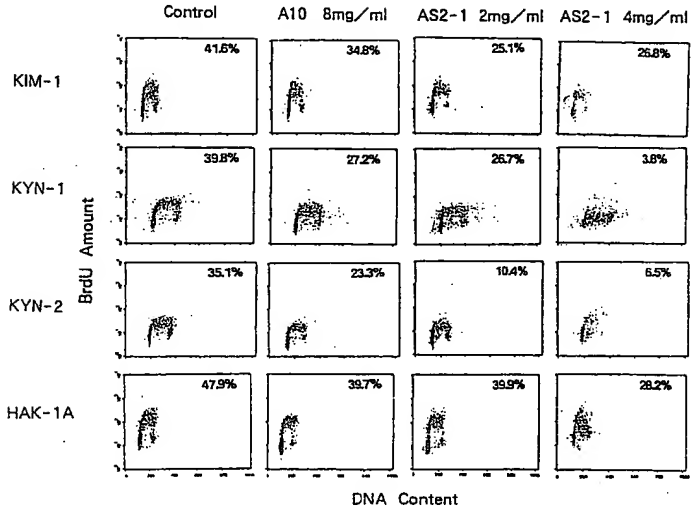

Fig. 3. Double stained flowcytometry of KIM-1, KYN-1, KYN-2, HAK-1A hepatocellular carcinoma cells treated with Antineoplaston A10 at concentration of 8 mg/ml and Antineoplaston AS2-1 at concentrations of 2, 4 mg/ml for 72 hs. Number in % indicates BrdU uptake. Antineoplaston A10 and AS2-1 reduced BrdU uptake ratio and number of cells in G2/M phase.

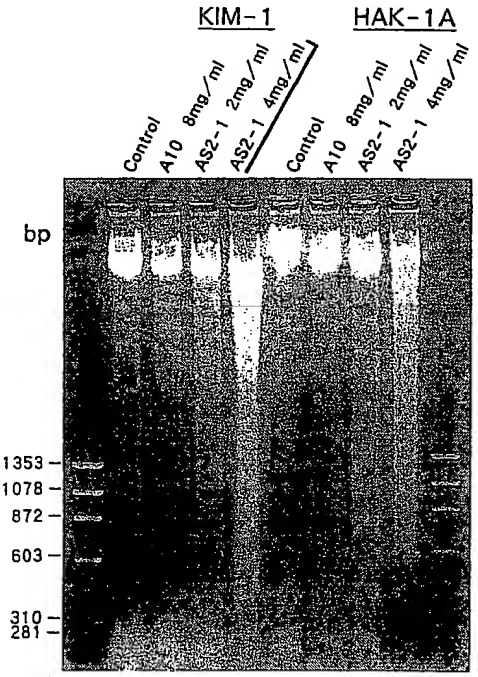

Fig. 4. Electrophoresed DNA of KIM-1, HAK-1A cells treated with Antineoplaston A10 at concentration of 8 mg/ml and Antineoplaston AS2-1 at concentrations of 2,4 mg/ml for 72 hs. Number indicates DNA molecular size marker. DNA fragmentation with ladder was observed in Antineoplaston AS2-1 treated cells.

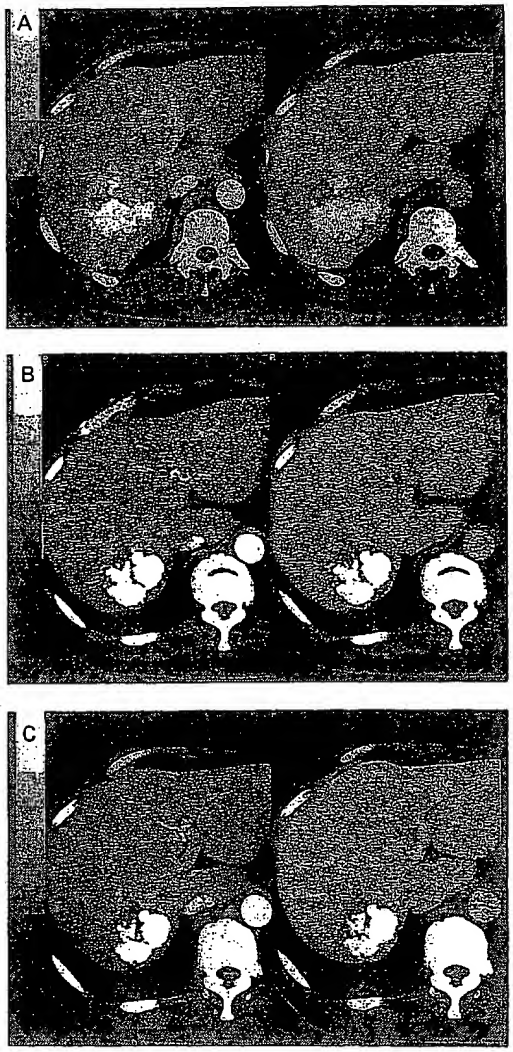

Fig. 5. Early and late phases of dynamic CT of liver of a hepatocellular carcinoma patient who had received Antineoplaston AS2-1 treatment. CT taken 3 months after the first incomplete TAE (A), CT taken 13 months after TAE (B), CT taken 20 months after TAE (C). A gradual reduction of viable lesion was observed.

Discussion

Hepatocellular carcinoma (HCC) is becoming a major problem in Japan (Okuda et al. 1987; Tanaka et al. 1990; Unoura et al. 1993). There is no doubt that early detection of a small solitary HCC by advanced diagnostic technology makes local approach such as PEIT, MCN, TAE, and surgery possible and contributes to better prognosis of this disease. However, even the patients treated in the early stage often show recurrence or multiple intrahepatic metastases (Ikeda et al. 1991; Nakao et al. 1991). This situation requires the intrahepatic arterial infusion of chemotherapeutic agents, these induce liver dysfunction since hepatocytes are essentially vulnerable to toxic agents and since chronic hepatitis and liver cirrhosis is almost always associated with HCC. Many patients in the terminal stage of this disease suffer from hepatic failure caused both by invasive tumors and by treatment with toxic agents. The development of less toxic agents that can prevent recurrence or multiple intrahepatic metastases after PEIT, TAE, MCN and surgery is crucial for overcoming this miserable situation.

Antineoplastons, naturally occurring peptides and aminoacid derivatives that control neoplastic growth, were first described by Burzynski in 1976. Antineoplaston A10 is the first of the antineoplastons to be chemically identified (Burzynski and Hai, 1985). Hendry speculated in a stereospecific study that Antineoplaston A10 (3-phenylacetylamino-2,6-piperidinedione) could intercalate between base pairs of DNA and predicted that this agent would have a

chemopreventive effect against carcinogens (Hendry and Muldoon,1987). Several authors have since reported the chemopreventive effect of this compound against various kinds of carcinogens (Eriguchi et al. 1988; Muldoon et al. 1988). Antineoplaston A10, when administered orally, is hydrolysed and degraded, to phenylacetylglutamine, phenylacetylisoglutamine, phenylacetic acids, glutamic acids.

A mixture of phenylacetylglutamine and phenyl acetic acid in the ratio of 1 to 4 (Antineoplaston AS2-1) was confirmed to have a antineoplastic and cell differentiating activities. Samid reported that Antineoplaston AS2-1 and phenylacetate, one of active components in Antineoplaston AS2-1, induced cell differentiation in erythroleukemia, fibrosarcoma, and malignant melanoma cells. (Samid et al. 1991, 1992). In our study, Antineoplaston A10 and AS2-1 exhibited dose- and time-dependent inhibitory effects on cell proliferation in all 6 HCC cell lines tested; both agents increased the cell population in G_0 and G_1 phases and reduced that in S phase, suggesting that they inhibited cell proliferation by arresting cell growth and not by destroying cells. The increase in cells in D_1 region with Antineoplaston AS2-1 treatment indicates that these cells were about to become apoptotic. The presence of apoptotic cells in the morphological study and the DNA fragmentation in the Antineoplaston AS2-1 treated cells would suggest that these cells stopped growing and died from apoptosis. Antineoplaston A10 inhibited cell proliferation but did not induced apoptosis. The difference between Antineoplaston A10 and AS2-1 is that AS2-1 contains phenylacetic acid. The role played by phenylacetic acid in inducing cell differentiation or apoptosis is unclear. Liau and Burzynski (1986) have speculated that the hypomethylation of nucleic acids induced by phenylacetylglutamine and phenylacetic acid is involved in these processes.

The activation of P53 by Antineoplaston AS2-1 probably occurs in hepatocellular carcinoma cells. Liau demonstrated that Antineoplaston AS2-1 converted the cancer methylation enzyme which is 7 times more active than normal to the normal enzyme, which would lead to the relative hypomethylation of nucleic acids and could change gene expression. Those speculations should be confirmed by direct evidence in future studies.

The continuous oral administration of Antineoplaston AS2-1 8-10 g/day in our patient induced gradual disappearance of the viable part of the tumor and no metastases of HCC was seen on dynamic CT taken at 13 and 20 months. Considering that most of HCC patients who have had TAE with HCC of this size develop recurrence and intrahepatic multiple metastases within a year, the dynamic CT findings in this particular patient would suggest that the viable tumor tissue around the incompletely embolized area first became stable and then disappeared after Antineoplaston AS2-1 administration. This clinical picture seems to be consistent with results of our in vitro study of Antineoplaston AS2-1. This patient has been working without any limitations on his normal activity, except when he was admitted to the hospital for TAE, PEIT for a short period.

Since HCC patients have underlying liver dysfunction, chemotherapy for recurrence or multiple intrahepatic metastases often leads to a miserable outcome. To improve treatment after the early detection of HCC, a less toxic antineoplastic and antitumorigenic approach should be developed. From this point of view, Antineoplaston AS2-1 appears to be one of the best candidates of drug for the treatment of HCC.

To summarize, we found that Antineoplaston A10 and AS2-1 exhibited an inhibitory effect on cell proliferation in 6 HCC cell lines, and both agents increasing the cell population in G_0 and G_1 phases. Antineoplaston AS2-1 induced apoptosis in HCC cells.

We also described the clinical case of a HCC patient who showed the gradual disappearance of a viable tumor lesion in 13 and 20 months after the beginning of Antineoplaston AS2-1 treatment.

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PRECLINICAL STUDIES ON ANTINEOPLASTON A10 INJECTIONS

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Summary: *Antineoplaston A10 (3-phenylacetylamino-2, 6-piperidinedione) has poor solubility in water. In order to make it more soluble for the preparation of Antineoplaston A10 injections, the compound has to be converted into its sodium salt. It was found that during neutralization A10 undergoes basic hydrolysis with formation of two components, IIa and IIb. However, A10 was found to be fairly resistant to acid hydrolysis at room temperature. At a higher temperature (110°C) the reaction proceeded easily and after 60 min the compound was completely hydrolysed. The ratio of 4 : 1 of products of basic hydrolysis remained very constant in a number of experiments. They were subsequently identified as phenylacetylglutamine and phenylacetylisoglutamine. A similar ratio of these two compounds was found during partial hydrolysis of A10 in simulated pancreatic juice, indicating a possibility that at least some of the anticancer effects of A10 are attributable to the action of these two degradation products. A decision was therefore made to produce a formulation of Antineoplaston A10 injections, 100 mg/ml as a 4 : 1 mixture of sodium salts of phenylacetylglutamine and phenylacetylisoglutamine. This formulation did not show any significant toxic effects when tested for one year in chronic toxicity studies in a group of 160 HA/1CR Swiss white mice.*

Introduction

Antineoplaston A10 (I) is a small peptide which was initially isolated from human urine (1, 2). The chemical structure of I corresponds to 3-phenylacetylamino-2, 6-piperidinedione. Oral formulation of I has been submitted for tissue culture, animal and human toxicology studies (3-6). The logical next step was the preparation of Antineoplaston A10 injections. 3-Phenylacetylamino-2, 6-piperidinedione has poor solubility in water; and in order to make it more soluble it has to be converted to its sodium salt. It was found, however, that the sodium salt of I was unstable and that it was hydrolysing into two main products. In view of that, a decision was made to study the fate of I when subjected to acidic and basic conditions and to identify the final products of hydrolysis. This paper describes these experiments as well as chronic animal toxicology and stability studies of Antineoplaston A10 injections.

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Materials and methods

Materials and analytic procedures

3-Phenylacetylamino-2, 6-piperidinedione was synthesized by the Department of Organic Chemistry of the Burzynski Research Institute, Inc. The pharmaceutical formulation of Antineoplaston A10 injections was prepared by the Department of Pharmacy of the same Institute. All reagents were of analytical grade.

Infrared spectra were run on a Perkin Elmer infrared spectrophotometer, Model No. 710 B. Proton NMR spectra were run on a Joel JMMFX 92 spectrometer using $(CD_3)_2CO$ as the solvent. HPLC analyses were performed on a Glenco Scientific, Inc. instrument equipped with a 5480 UV monitor set at 254 nm, AUFS: 05, a μ Bondapak C-18 (Waters, 3.9 mm x 30 cm) column, an omniscriber recorder and a Hewlett Packard Integrator, No. 3390A. The flow rate was 0.8 ml/min using water : methanol : glacial acetic acid, v/v 85 : 15 : 0.1 as the eluent or as indicated. Amino acid analyses were performed on a Glenco Model "MM" series amino acid analyser equipped with a Glenco model DP 810 Digital Programmer, a Hewlett Packard Integrator No. 3390A and a sulfonated polystyrene column (0.325 x 30 cm). The absorption peaks were automatically recorded and integrated. A solution of standard amino acids was used as a reference.

Basic hydrolysis of I. To a stirred slurry of I (6.15 g, 25 mmoles) in 60 ml of deionized water, a solution of sodium hydroxide (1 g, 25 mmoles in 8 ml of water) was added dropwise. The pH of the solution was measured after each addition of 1/4, 1/2, 3/4, and 1 equivalent of the base. When neutralization was achieved after each portion of the base was added, small aliquots (0.4 ml) were withdrawn from the reaction mixture. The aliquots were filtered, lyophilized and then redissolved in dimethylformamide for HPLC analysis.

Acid hydrolysis of I. The samples of 2 mg of I and 20 μl of 12N hydrochloric acid were introduced into nine ampules and eight of them were heated at 110°C for different time intervals. The remaining ampule was kept at room temperature for 90 min. The results of acid hydrolysis at 110°C were studied after 10, 20, 30, 50, 60, and 90 min and 16 h. After hydrolysis, the seals of all ampules were broken and the ampules were left in a desiccator containing sodium hydroxide and calcium chloride pellets for removal of hydrochloric acid. The dried residues were redissolved in dimethylformamide and analysed by HPLC.

Organic synthesis procedures

Synthesis of phenylacetylglutamine. L-glutamine (440 g, 3.0 mole) and sodium bicarbonate (900 g, 10.7 mole) was added to 13 l of deionized water in a glass reactor equipped with a mechanical stirrer. After stirring for 5 min, four 133.75 ml portions of phenylacetyl chloride (535 ml, 4.01 mole) were added to the reactor, one portion every 15 min, and further stirred for 30 min. The pH of the reaction was brought to 2.5 with concentrated hydrochloric acid. The reaction mixture was extracted with a 1400 ml portion of methylene chloride three times. The water layer was transferred to a Nalgene tank and pH was adjusted to 7.0 by adding 12N sodium hydroxide. The solution was concentrated on a rotary evaporator to 3.5 l. The pH of the solution was adjusted to 2.0 with concentrated hydrochloric acid. Ethyl acetate (4 l) was added and the solution stirred vigorously until phenylacetylglutamine started to precipitate. The precipitate was filtered and again suspended in ethyl acetate (4 l) and stirred for 1 h. Filtering the precipitate and freeze drying yielded 490 g of phenylacetylglutamine with 91% purity.

A 10 g sample of phenylacetylglutamine was purified by extraction with ethyl acetate on a continuous extractor. A crystallization from ethyl acetate yielded 7.5 g of pure phenylacetylglutamine (m.p.

155°C; IR Nujol Mull: 3380 cm^{-1}, 3300 cm^{-1}, 3200 cm^{-1}, 1700 cm^{-1}, 1660 cm^{-1}, 1530 cm^{-1}, 1450 cm^{-1}, and 1240 cm^{-1}; proton NMR: δ7.25 (m, H, arom.), 4.63 (br.s, NH), δ4.45 (sextet NCH), δ2.61–δ1.64 (complex m, $PhCH_2$, CH_2CO_2H, CH_2CONH_2, $CHCH_2$).

Synthesis of phenylacetylglutamic acid. L-glutamic acid (117.68 g, 0.8 mole) was suspended in a mixture of water (4 l) and acetone (2 l). Subsequently, sodium bicarbonate (336.0 g, 5 eq.) was added in four 42 ml portions, each at 15 min intervals. The solution was stirred for 1 h and extracted three times with 2 l of methylene chloride to remove acetone. The pH of the solution was adjusted to 2.0 with 3N hydrochloric acid (1200 ml) and again extracted with methylene chloride (2 l). The concentration of the water layer on a rotary evaporator to 600 ml resulted in formation of precipitate. Filtration and drying of the precipitate yielded 141.8 g (66.83%) of phenylacetylglutamic acid (m.p. 122–123°C; IR: 3300 cm^{-1}, 3340 cm^{-1}, 1730 cm^{-1}).

Synthesis of phenylacetylisoglutamine. Phenylacetylglutamic acid (10 g, 37.7 mmoles) was suspended in a mixture of 400 ml of xylene, 200 ml of benzene and 9.4 g of paraformaldehyde. Subsequently, 1 g of p-toluenesulfonic acid was added. The mixture was refluxed for 1 h with azeotropic water removal. The solution was freeze dried. The residue was dissolved in 300 ml of ethyl acetate, washed with 200 ml of water and dried over sodium sulfate. The resulting solution was concentrated *in vacuo* and afforded 8.7 g of (S)-3-phenylacetyl-5-oxo-4-oxazolidinonepropionic acid.

The oxazolinone was used without further purification. It was dissolved in 100 ml of ethyl alcohol and 7.6 ml (4 eq.) of liquid ammonia. The solution was allowed to stand at room temperature for 48 h. After evaporation, 10% hydrochloric acid was added dropwise to the residue and the crystalline product was filtered. The recrystallization from ethyl acetate yielded 4.6 g of phenylacetylisoglutamine, in 46.17% yield; m.p. 145–147°C; IR Nujol Mull: 3450 cm^{-1}, 3325 cm^{-1}, 3200 cm^{-1}, 1740 cm^{-1}, 1680 cm^{-1}, 1600 cm^{-1}, 1550 cm^{-1}, 1460 cm^{-1}, 1420 cm^{-1}, 1180 cm^{-1}, 1030 cm^{-1}, and 800 cm^{-1}; proton NMR: δ7.5 (m, H, arom.), δ3.36 (br S, NH), δ4.45 (sextet, NCH), δ2.51–δ1.51 (complex m, $PhCH_2$, CH_2CO_2H, CH_2CONH_2, $CHCH_2$).

Stability studies on Antineoplaston A10 injections

Stability testing of Antineoplaston A10 injections was done according to the standard protocol of Burzynski Research Institute, Inc. Three different batches of Antineoplaston A10 injections were stored at three different temperatures: 5°C, 25°C, 60°C. The samples were tested at the following time intervals: 1, 2, 3, 4, 5, 6, 9, 12, and 15 months from the beginning of the stability testing. Each sample was tested twice for determination of dry weight, incombustible residue, pH, heavy metal content, and HPLC analysis before and after acid hydrolysis. Each sample was also tested for acute toxicity in a group of eight HA/ICR Swiss white mice. A dose of 0.5 ml of formulation of Antineoplaston A10 injections, 100 mg/ml, was injected intraperitoneally into the left lower quadrant of the abdomen. The animals were observed for one week after the injection.

Chronic toxicity studies on Antineoplaston A10 injections

The experiments were carried out in HA/ICR Swiss white mice, approximate weight 22 g. A total of 160 mice were used. The animals were divided in four groups, each group containing 20 male and 20 female mice. The first group received 92.3 mg/kg, the second 553.8 mg/kg and the third 1107.6 mg/kg of Antineoplaston A10 injections intraperitoneally daily. The fourth group served as the control. The animals were sacrificed on days 30, 60, 90, 180 and

365. Four of the mice sacrificed on each day belonged to the injected group and four to the control group. The animals underwent complete physical examination, autopsy, and microscopic examination of major organs such as brain, heart, lungs, gastrointestinal tract, liver, spleen, kidneys, and thymus.

Results

Basic hydrolysis of Antineoplaston A10. HPLC analysis of hydrolysis indicated the presence of two components in the approximate ratio 4:1. These components were initially present in the form of sodium salts IIa and IIb. After passing through a HPLC column, IIa and IIb converted into acid forms IIIa and IIIb (Table I) due to the presence of acetic acid in the eluent. IIIa was later identified as phenylacetylglutamine and IIIb as phenylacetylisoglutamine. The above experiment was repeated in 40 ml and 100 ml of water but the ratio of the products still remained unchanged. In all cases the total time required for complete neutralization of I was approximately 2 h.

Isolation, purification, and identification of IIIa and IIIb. The solution of sodium salts of hydrolysis products IIa and IIb (58.2 g, 200 mmoles) in 200 ml of water was treated with 2N hydrochloric acid until pH 2.5. After leaving overnight at room temperature the solid was filtered and both solid and filtrate analysed by HPLC. The solid, which was found to be rich in component IIIa by HPLC analysis, was recrystallized several times with hot water and yielded 20 g of pure IIIa (m.p. 155 °C). Retention time of this pure component was 18.80 min, which was identical to that of synthetically prepared phenylacetylglutamine.

The initial filtrate which was a 1:1 mixture of IIIa and IIIb was continuously extracted with 600 ml of ethyl acetate for 6 h. After leaving the extract at room temperature overnight, the precipitated solid was filtered. This filtrate was found to be rich in minor product IIIb by HPLC analysis. It was concentrated to become a cloudy solution, which was left overnight at room temperature. The precipitated solid was filtered. Several recrystallizations from ethyl acetate gave 110 mg of pure IIIb, (m.p. 145–147 °C). HPLC analysis of this pure component indicated a retention time of 22.71 min, which was identical to that of phenylacetylisoglutamine prepared synthetically. IR and NMR spectroscopic data of IIIb were identical to synthetically prepared phenylacetylisoglutamine.

Table I *Basic hydrolysis of Antineoplaston A10.*

Equivalent of sodium hydroxide added	Retention time (min.)		Ratios IIIa:IIIB
	IIIa Phenylacetyl-glutamine	IIIb Phenylacetyl-isoglutamine	
1/4	15.50	18.88	4:1
1/2	15.42	17.78	4:1
3/4	15.45	18.94	4:1
1	15.61	18.85	4:1

Acid hydrolysis of Antineoplaston A10. HPLC analysis indicated that acid hydrolysis of I was complete within 60 min, with the intermediate formation of product IV which had a retention time 13.27 min, identical to that of phenylacetylglutamic acid prepared synthetically. After 60 min of hydrolysis a single component V was detected which had a retention time of 23.47 min, identical to that of synthetically prepared phenylacetic acid. The acid hydrolysis products of 60 min were lyophilized to remove DMF and the dried residue was redissolved in water (4 ml). Amino acid analysis was performed on 0.1 ml of this hydrolysate. Only one amino acid (VI) was detected by the analyser; it had a retention time identical to that of standard glutamic acid (18.81 min). Ammonia was also detected (Table II).

Table II *Acid hydrolysis of Antineoplaston A10.*

Sample	Hydrolysis time (min)/temp. (°C)	Retention time					Ratios				
		I	IIIa	IIIb	IV	V	I	IIIa	IIIb	IV	V
I	90/rt	12.22	-	-	-	-	1	0	0	0	0
I	10/110	12.02	9.75	10.62	13.80	-	2.5	1	2.2	2.4	0
I	20/110	12.17	9.89	10.78	13.89	24.48	8.3	1	1	5.2	1.6
I	30/100	12.05	9.75	10.65	13.60	24.27	7.2	1.2	1	10.7	4.0
I	40/110	12.05	-	-	13.47	24.02	1	0	0	22.4	17.0
I	50/100	-	-	-	13.65	24.03	0	0	0	1	2.1
I	60/110	-	-	-	-	23.97	0	0	0	0	1.0
I	90/110	-	-	-	-	23.98	0	0	0	0	1.0
I	16 hours/110	-	-	-	-	23.03	0	0	0	0	1.0
30 min + IV synthet. prepared		11.95	9.68	10.57	13.27	23.96	6.8	1	1	18.2	4.4
Standard synthet. prepared		12.29	9.72	-	-	24.04	-	-	-	-	-

Acid hydrolysis of IIIa, IIIb and IV. Acid hydrolysis of IIIa, IIIb and IV, which were isolated from the reaction mixture of I and synthetically prepared IIIa, IIIb and IV, was performed for 4 h at 110 °C. HPLC analysis (water : methanol : glacial acetic acid v/v 50 : 50 : 0.1) of each indicated the presence of a single component with a retention time of 9.25 min, identical to that of phenylacetic acid. Similarly the presence of a single amino acid with a retention time of 18.81 min, identical to that of standard glutamic acid, and another peak corresponding to ammonia was detected by the amino acid analyser.

Formulation of Antineoplaston A10 injections. It is apparent from the above results that two compounds, phenylacetylglutamine and phenylacetylisoglutamine, are formed during dissolving and neutralization of I. The ratio of 4 : 1 of these two components remained constant in a number of experiments. According to pharmacokinetics studies of I administered orally, there was no substantial hydrolysis of I in simulated gastric juice, but 30% of I was hydrolysed when exposed to simulated pancreatic juice for 3 h. The products of hydrolysis corresponded to phenylacetylglutamine and phenylacetylisoglutamine in a ratio of 3:1 (7). In view of these findings it was decided to formulate Antineoplaston A10 injections, 100 mg/ml, as the mixture of sodium salts of phenylacetylglutamine and phenylacetylisoglutamine in a ratio of 4:1.

Stability studies on Antineoplaston A10 injections. Three batches of Antineoplaston A10 injections were stored at 5 °C, 25 °C and 60 °C for various periods up to 15 months. Compounds I, IIIa and IIIb are nonreactive with ninhydrin, whereas their degradative products, namely, glutamine, glutamic acid and ammonia, are reactive with ninhydrin. These three degradative products can be identified by amino acid analyser with retention times of 16 min, 19 min and 72 min respectively. Chemical stability tests were based on the detection of these degradative products as well as UV absorbing components resolved by C-18 HPLC. Analytical results indicated that the peptide bond was very stable. The amide bond was also stable at 5 °C and 25 °C, since the amounts of ammonia in the specimens stored at 5 °C and 25 °C for various periods

up to 15 months remained approximately the same as in the zero time specimens. However, there were progressive increases of ammonia in the specimens stored at 60 °C, corresponding to the hydrolysis of 0.1 to 0.2% of the amide of Antineoplaston A10 components per month. The pH dropped slightly in the specimens stored at 60 °C, reflecting perhaps the hydrolysis of the amide bond.

C-18 HPLC analyses revealed that the total amounts of the two major Antineoplaston A10 injection components (IIIa and IIIb) remained approximately the same, and that phenylacetic acid was not detectable. The preservative methylparaben, which has a retention time of 65 min, was stable at 5 °C and 25 °C. At 60 °C a new component with a retention time of 19.5 min appeared at the expense of methylparaben. This component was most likely p-hydroxybenzoic acid, the degradation product of methylparaben. These studies enable us to conclude that Antineoplaston A10 injections are very stable preparations. The chemical constituents remain essentially unchanged for up to 15 months, if stored at temperatures below 25 °C. At 60 °C a limited amount, approximately 0.1 to 0.2% per month, of amide may be hydrolysed. Methylparaben is also unstable at 60 °C.

Chronic toxicity studies on Antineoplaston A10 injections. Chronic toxicity studies did not show any significant toxicity. Physical examination, gross autopsy and microscopic examination of the organs of mice receiving Antineoplaston A10 injections were similar to those of the control animals. The exception was the presence of bronchial adenoma in the control group.

Discussion

The results of basic hydrolysis of I (Table I) indicate that the ratio of the two products remained very constant, i.e., approximately 4:1, even as the concentration of the product is increased with the disappearance of the starting material. These ratios were not affected by decreasing or increasing the amount of water used for neutralization. Isolation and identification of the reaction products by spectroscopic and HPLC methods indicated that the major product IIIa had properties identical to those of phenylacetylglutamine and the minor component IIIb had properties identical to those of phenylacetylisoglutamine prepared synthetically. From the identification of these reaction products it seems likely that neutralization of I with one equivalent of base proceeds as shown in Fig. 1.

Electron attracting groups are known to accelerate hydrolysis. Therefore, one would expect the acyl carbon 2 of I to be more susceptible to nucleophilic attack by the base than the acyl carbon 6. Since acyl carbon 2 is in a closer vicinity of the carbon 3 bearing another amide group, it leads to the formation of IIIa as the major component. The results of acid hydrolysis suggest that the reaction proceeds by the mechanism shown in Fig. 2.

The detection of phenylacetylglutamic acid (IV) by HPLC indicates that in the first step of hydrolysis of I, a cleavage of the cyclic imide and not the secondary amide is taking place. The hydrolysis occurred at both acyl carbons 2 and 6, leading to the formation of the intermediate IIIa and IIIb (Table II). Since the conversion of IIIa and IIIb to IV was fast, it could not be determined which of the two was the major intermediate. Acid hydrolysis of the secondary amides is known to be difficult. Therefore, one would expect the hydrolysis of the secondary amide group of I to be the second step of reaction, leading to the formation of V and VI. This can also be illustrated by Table II; i.e., during the end of the reaction (50 min) only two products IV and V were detected by HPLC and after 60 min IV was completely hydrolysed to V and VI.

The results of the experiments indicate that 3-phenylacetylamino-2, 6-piperidinedione is fairly resistant to acid hydrolysis at room temperature. At a higher temperature (110 °C), however, the reaction proceeds easily and after 60 min of exposure to

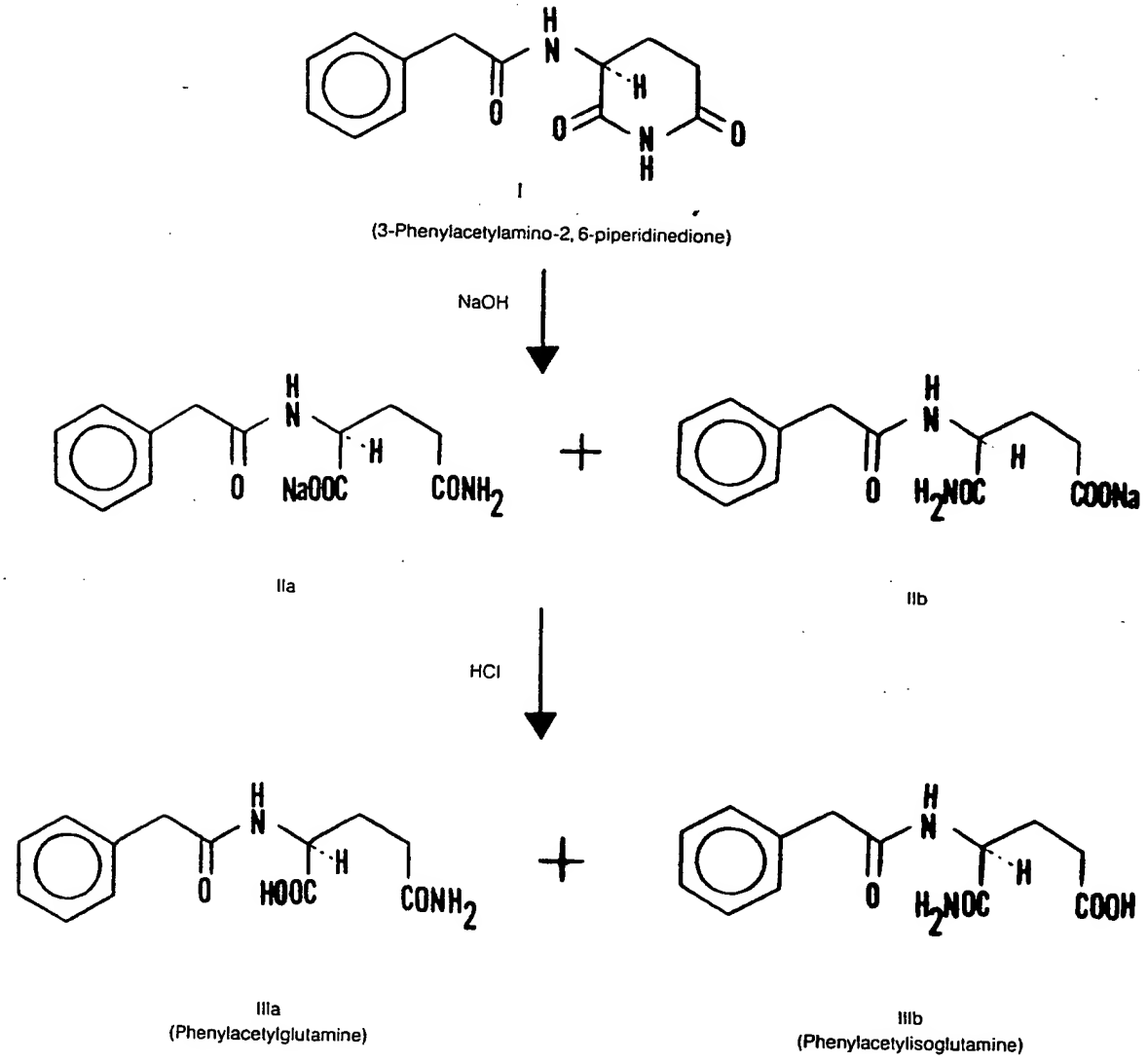

Fig. 1 Basic hydrolysis of Antineoplaston A10.

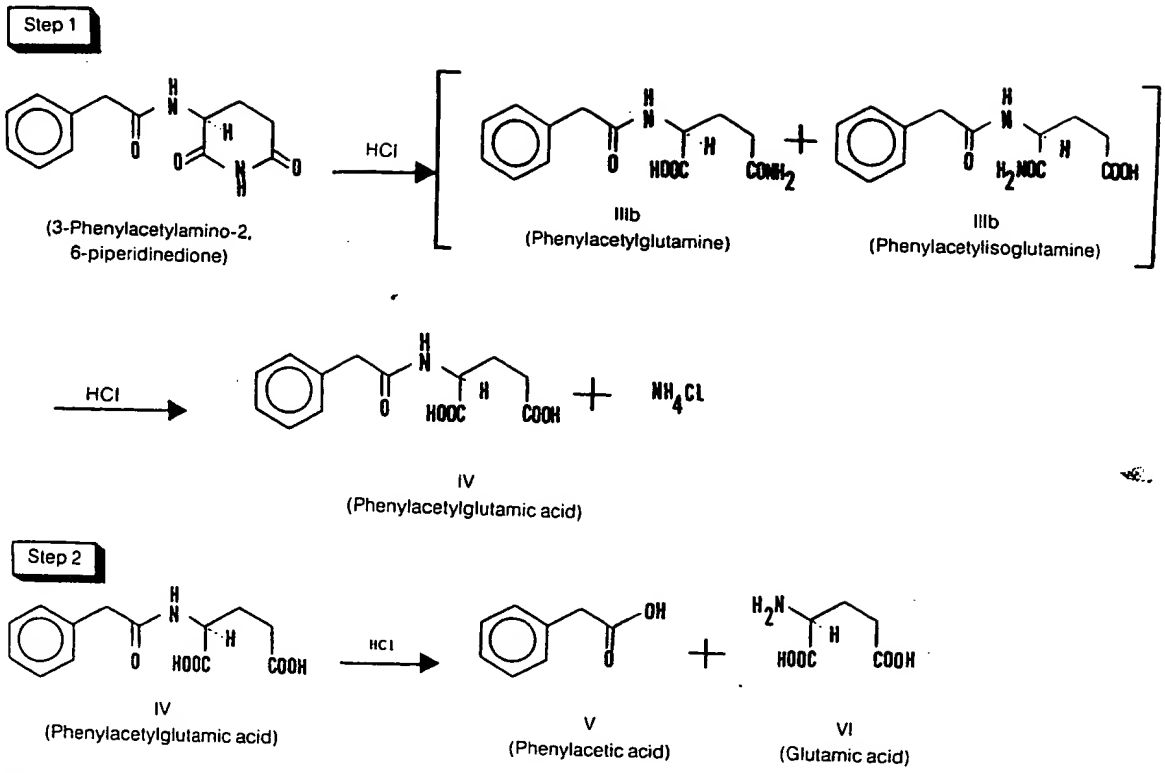

Fig. 2 Acid hydrolysis of Antineoplaston A10.

12N hydrochloric acid the compound is completely hydrolysed. On the other hand, this substance is easily hydrolysed under alkaline conditions.

The main products of hydrolysis include: phenylacetylglutamine, phenylacetylisoglutamine, phenylacetic acid and glutamic acid. Phenylacetylglutamine and phenylacetic acid have been studied by the authors' group in tissue culture, animal toxicology in Phase I clinical studies (8–12). The pharmaceutical formulation of phenylacetylglutamine was named Antineoplaston AS2-1. Both formulations have low toxicity and have shown promising clinical results, including complete remission with a five year disease-free period in advanced cancer patients.

Antineoplaston A10 administered orally has been shown to produce promising clinical results (4). Antineoplaston A10 was partially hydrolysed in a simulated pancreatic juice to products similar to alkaline hydrolysis products (7). Therefore, when Antineoplaston A10 is administered orally, at least part of the compound is broken down to phenylacetylglutamine and phenylacetylisoglutamine as a result of digestion with pancreatic juice. It is possible that at least some anticancer effects of Antineoplaston A10 are attributable to the action of these two degradation products. This is the main reason why the decision was made to produce a formulation of Antineoplaston A10 injections, 100 mg/ml, as a mixture of sodium salts of phenylacetylglutamine and phenylacetylisoglutamine in the ratio of 4:1. Although Antineoplaston A10 is unstable under alkaline conditions, the two initial hydrolysis products, namely phenylacetylglutamine and phen-

ylacetylisoglutamine, are very stable at neutral pH and free from significant toxicity.

Antineoplaston A10 injections have been submitted for phase I clinical studies in cancer patients. The results of such studies are currently being evaluated.

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